EFFECTS OF GENISTEIN AND DAIDZEIN ON ARTERIAL TONE AND BLOOD PRESSURE IN RATS

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Academic Dissertation

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I Nevala R, Korpela R, Vapaatalo H (1998). Plant derived estrogens relax rat mesenteric artery *in vitro*. *Life Sci* 63:95-100.

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IV Nevala R, Vaskonen T, Vehniäinen J, Korpela R, Vapaatalo H (2000). Soy based diet attenuates the development of hypertension when compared to casein based diet in spontaneously hypertensive rat. *Life Sci* 66:115-124.

V Nevala R, Lassila M, Finckenberg P, Paukku K, Korpela R, Vapaatalo H: Genistein treatment reduces arterial contractions by inhibiting tyrosine kinases in ovariectomized spontaneously hypertensive rats (SHR). *J Vasc Res* (Submitted).

MAIN ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
Ang I	Angiotensin I
Ang II	Angiotensin II
4-AP	4-aminopyridine
ChTX	Charybdotoxin
EDHF	Endothelium-derived hyperpolarizing factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelin nitric oxide synthase
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERT	Estrogen replacement therapy
ET-1	Endothelin-1
HDL	High density lipoprotein
IbTX	Iberiotoxin
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Ca^{2+}-activated K^+ channel
K_{IR}	Inward rectifier K^+ channel
K_V	Voltage-dependent K^+ channel
LDL	Low density lipoprotein
L-NAME	N^G-nitro-L-arginine methyl ester
LVH	Left ventricular hypertrophy
OVX	Ovariectomy
PGI_2	Prostacyclin
SHR	Spontaneously hypertensive rat
SNP	Sodium nitroprusside
TEA	Tetraethylammonium
VSMC	Vascular smooth muscle cell

ABSTRACT

Estradiol-17β lowers blood pressure and dilates arteries. Genistein and daidzein are plant-derived estrogens which originate mainly from soybean. Genistein and daidzein have been shown to lower serum cholesterol values, but their influence on other cardiovascular risk factors is still mainly unclear. The present series of studies was carried out to investigate the effects of genistein and daidzein on arterial tone and blood pressure, and to compare these effects with those of estradiol-17β. The study was focused on the following mechanisms of action of genistein and daidzein: gender, endothelium, potassium channels (K^+ channels), estrogen receptors (ERs), and the inhibition of tyrosine kinases. Male, female and ovariectomized (OVX) normotensive Wistar rats, and male, female and OVX spontaneously hypertensive rats (SHR) were used.

In the rat mesenteric arteries, estradiol-17β, genistein and daidzein induced relaxation gender- and endothelium-independently, estradiol-17β being the most potent relaxant and daidzein the weakest. Tamoxifen, an antagonist of the estrogen receptor α (ERα), did not inhibit estradiol-17β-, genistein- or daidzein-induced relaxations. Estradiol-17β- and daidzein-induced relaxations were inhibited by iberiotoxin (IbTX), an inhibitor of large conductance K_{Ca} channels, and by apamin, an inhibitor of small conductance K_{Ca} channels. Genistein-induced relaxation was also inhibited by IbTX, but not by apamin.

In the *ex vivo* studies, mesenteric arterial contractility was more prevalent among males and OVX normotensive rats. The five-week soy protein supplementation had no effect on the contractions in either male or female SHRs.

The two-day low-dose genistein (2.5 mg/kg/d) treatment attenuated renal arterial contractility in OVX SHRs, but the estradiol-17β (25 μg/kg/d) and the high-dose genistein (25 mg/kg/d) treatments did not. The two-week treatments had no effect on renal arterial contractility in OVX SHRs. The two-day low-dose genistein treatment reduced tyrosine phosphorylation in aortic smooth muscle cells of OVX SHRs, whereas the two-day treatments of estradiol-17β and high-dose genistein, as well as the two-week treatments of low-dose genistein, high-dose genistein or estradiol-17β, did not alter the tyrosine phosphorylation.

Male gender and OVX in normotensive rats decreased endothelium-independent relaxations, whereas the soy protein supplementation in SHRs, the low- (2.5 mg/kg/d) and the high-dose (25 mg/kg/d) genistein, and the estradiol-17β (25 μg/kg/d) treatments in OVX SHRs had no effect on either endothelium-dependent or –independent arterial relaxations.

A five-week supplementation with soy protein, rich in genistein and daidzein, attenuated the development of hypertension in SHRs compared to a casein-based diet. The two-week estradiol-17β, and the low-dose or high-dose genistein treatments had no effect of the development of hypertension in OVX SHRs.

From the present results it can be concluded that the plant-derived estrogens genistein and daidzein have relaxing effects similar to estradiol-17β on arterial smooth muscle in rats *in vitro*. These relaxations are independent of ERα, endothelium and gender, but are related to the activation of K_{Ca} channels. The tyrosine kinase inhibition of genistein also plays a role in genistein-induced alterations in arterial tone. The soy protein, rich in genistein and daidzein, has an attenuating effect on the development of hypertension in SHRs. The possible role of genistein and daidzein as alternatives to estradiol-17β in protection against cardiovascular diseases remains to be clarified in clinical studies.

1. INTRODUCTION

Plant-derived estrogens are plant substances that are structurally or functionally similar to estradiol-17β or that produce an estrogenic effect (for review, see Fowler 1983). Plant-derived estrogens bind competitively to both estrogen α (ERα) and estrogen β (ERβ) receptors and activate them (Kuiper *et al.* 1997). Genistein and daidzein are plant-derived estrogens which originate from soybean (Eldridge & Kwolek 1983). Genistein and daidzein have been shown to protect against menopausal symptoms, osteoporosis and hormone-dependent cancers (for review, see Adlercreutz & Mazur 1997).

In the premenopause, women have less cardiovascular diseases than men of the same age. After the menopause, the risk of these diseases increases. Postmenopausal estrogen replacement therapy (ERT) reduces this risk by 50% (Barret-Connor & Bush 1991). For many years the favourable effects of ERT on serum lipid values were considered to be the only factor in the decreased risk of cardiovascular diseases in postmenopausal women. ERT reduces serum total cholesterol, LDL (Nabulsi *et al.* 1993) and triglycerides (Bongard *et al.* 1998), and increases HDL (Nabulsi *et al.* 1993). Nowadays, it is estimated that only about 20-50% of the lowered risk is due to changes in lipid metabolism (Nasr & Breckwoldt 1998).

Hypertension is an important risk factor for cardiovascular diseases such as stroke and atherosclerosis. ERT reduces blood pressure in postmenopausal women (Luotola 1983; Szekacs *et al.* 2000). It also prevents the development of hypertension in various rat models such as the normotensive ovariectomized (OVX) Sprague-Dawley rat (Brosnihan *et al.* 1994), and normal (Iams & Wexler 1979; Williams *et al.* 1988) and OVX (Iams & Wexler 1979) female spontaneously hypertensive rats (SHR). SHR is an inbred strain, which develops hypertension with increasing age. SHR is a widely studied and probably the best animal model for human essential hypertension.

The endothelium plays an essential role in regulating arterial tone and platelet aggregation, by secreting vasodilating substances such as nitric oxide (NO), prostacyclin (PGI_2), and an endothelium-derived hyperpolarizing factor (EDHF) in response to physiological stimuli and mediators such as histamine and bradykinin. ERT protects the function of the endothelium. Although estradiol-17β relaxes arterial smooth muscle

endothelium-independently (Mügge *et al.* 1993), both oral ERT (Lieberman *et al.* 1994) and estradiol-17β infusion (Gilligan *et al.* 1994) improve endothelium-dependent vasodilation in healthy postmenopausal women. In female SHRs, estrogen treatment enhances endothelium-dependent relaxations of the aorta (Williams *et al.* 1988). ERT reduces the synthesis of endothelium-derived contracting factors (Dantas *et al.* 1999) and maintains NO synthesis in OVX SHRs (Huang *et al.* 1997).

The tone of vascular smooth muscle cells (VSMCs) is controlled by the Ca^{2+} and K^{+} channels. When the Ca^{2+} channels open, the VSMC depolarizes and contracts. At the same time K^{+} channels open and K^{+} effluxes outside the cell. This causes hyperpolarization. It has been reported that the relaxing mechanism of estradiol-17β is related to the activation of K^{+} channels (White *et al.* 1995).

Genistein inhibits tyrosine kinase by interacting with the ATP-binding site, whereas daidzein is inactive in this respect (Akiyama *et al.* 1987). The inhibitors of tyrosine kinases have been shown to reduce VSMC contractions (for review, see Hughes & Wijetunge 1998). On the other hand, the substances which increase tyrosine phosphorylation in VSMC also induce contraction (Laniyonu *et al.* 1994).

It is known that the plant-derived estrogens genistein and daidzein have beneficial effects on serum lipids (Anderson *et al.* 1995), but their effects on the cardiovascular system are still poorly understood. The aim of this study was to investigate the effects of genistein and daidzein on arterial tone and blood pressure in normotensive rats, and in male, female and OVX female SHRs, and to compare these effects with those of estradiol-17β. The following mechanisms of the action of plant-derived estrogens were studied: gender, endothelium, potassium channels, estrogen receptors, and the inhibition of tyrosine kinase.

2. REVIEW OF THE LITERATURE

2.1. Plant-derived estrogens

Plant-derived estrogens are substances which originate from plants and produce estrogenic effects, or are structurally or functionally similar to estradiol-17β (for review, see Fowler 1983). The estrogen-like activity of plant-derived estrogens was discovered because there were reports in Australia of reduced fertility in female sheep fed on fresh clover (Moule *et al.* 1963). Plant-derived estrogens are classified as lignans, isoflavones, and coumestans (Table 1).

Table 1. Classification of plant-derived estrogens according to Korpela (1995).

Class	Examples	Main sources
Lignans	Enterolactone	Oilseed
	Enterodiol	Linseed
		Cereal bran
		Whole cereals
		Vegetables
		Fruits
		Legumes
Isoflavones	Genistein	Soybean
	Daidzein	Clover
	Equol	
Coumestans	Coumestrol	Clover

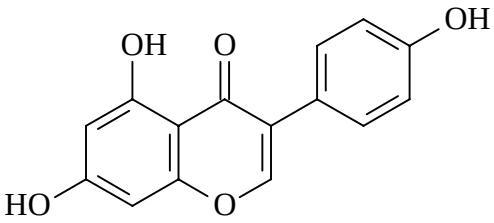
Plant-derived estrogens bind competitively to both estrogen α (ERα) and estrogen β (ERβ) receptors and activate them (Kuiper *et al.* 1997). ERα is called a classic ER and has been known for decades, while ERβ was described and characterized for the first time only a few years ago (Mosselman *et al.* 1996). Of all plant-derived estrogens, coumestrol has the most potent estrogen-like effect (Kuiper *et al.* 1997) (Table 2). It binds to ERα with a ten times lower affinity than estradiol17β, but its dissociation is close to that of estradiol-17β (Scarlata & Miksicek 1995). Coumestrol, however, is seldom found in the human diet. The methoxy derivative of genistein, biochanin A, does not bind ER, but is estrogenic *in vivo* (Miksicek 1994). Daidzein has a higher binding affinity to ER than its methoxy derivative, formononetin (Shutt & Cox 1972). It has been suggested that hydroxylation is necessary for a flavonoid to have estrogenic activity (Miksicek 1995). The flavonoids with hydroxyl substituents at 4' and 7 positions are estrogenic, and an additional hydroxyl group at the 5 position – like that possessed by genistein – increases estrogenic activity (Miksicek 1995). On the other hand, if a flavonoid has more than four hydroxyl substituents, (such as flavonol quercetin) or has a 4'-methoxylated substituent (such as hesperitin), the estrogenic activity is abolished (Miksicek 1995).

Table 2. Relative binding affinity of various compounds to ERα and ERβ, according to Kuiper *et al.* (1997).

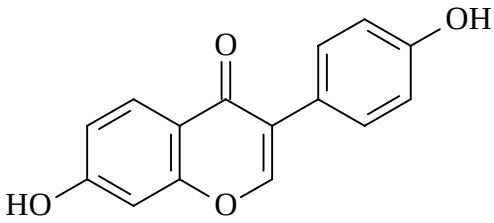
Compound	**ERα**	**ERβ**
Estradiol-17β	100	100
Estrone	60	37
Estriol	14	21
Progesterone	<0.001	<0.001
Testosterone	<0.01	<0.01
Coumestrol	94	185
Genistein	5	36
β-Sitosterol	<0.001	<0.001
Tamoxifen	7	6

2.1.1. Isoflavones

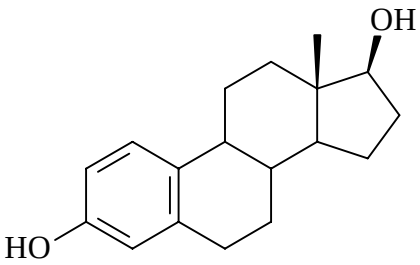
Soy products are widely consumed throughout the world. In Asian countries, soy has been an important part of the diet for more than a thousand years. Soy is the main source of the isoflavones genistein and daidzein (Figure 1) (Eldridge & Kwolek 1983). The consumption of soy products is estimated to be highest among the Japanese population, with the levels of isoflavones reaching 200 mg/day (Cassidy *et al.* 1994). In other parts of Asia, the diet provides 25-40 mg of total isoflavones per day, whereas in the western countries less than 5 mg/day is consumed (Coward *et al.* 1993).



Genistein



Daidzein



Estradiol-17β

Figure 1. Structural formulas of, genistein, daidzein and estradiol-17β.

Plant-derived estrogens, including isoflavones, are present in food as glycones, which are separated from other structural and storage components of the foods by the hydrolytic enzymes of gut bacteria (Setchell *et al*. 1982; Korpela 1995). In addition to the digestion of isoflavones from their glucose-containing precursors daidzin and genistin, daidzein may also be derived from formononetin and genistein from biochanin A. A significant portion of daidzein is further metabolized to equol, and genistein to p-ethylphenol (Figure 2). A major

proportion of plant-derived estrogens is excreted by the kidneys (for review, see Anderson & Garner 1997; Bingham *et al.* 1998). In humans, after a single soy meal the isoflavone concentrations rise slowly and reach maximum values of micromolar range at 7-8 hours (King & Bursill 1998). Genistein and daidzein have been detected in human plasma (Adlercreutz *et al.* 1994), urine (Adlercreutz *et al.* 1991), and milk (Franke & Custer 1996), as well as in saliva, breast aspirate and prostatic fluid (Finlay *et al.* 1991).

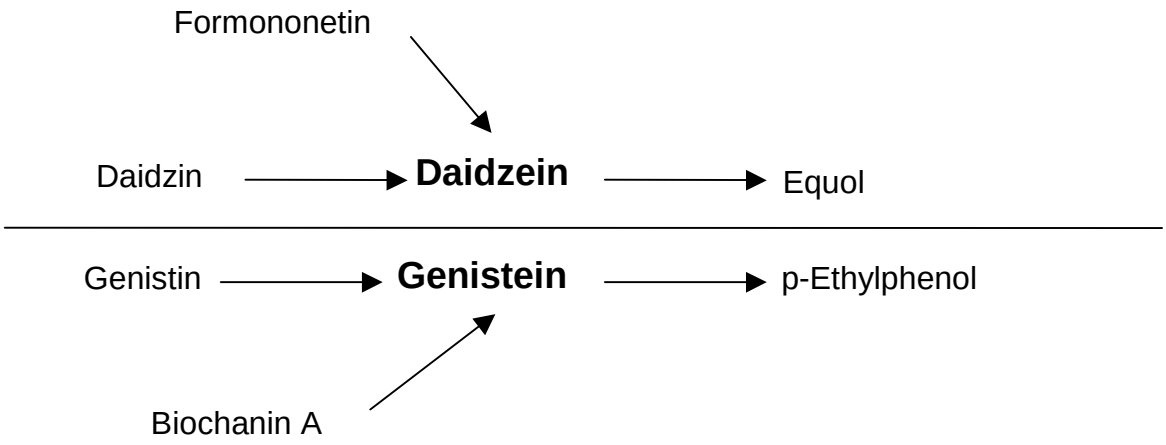


Figure 2. Intestinal metabolism of isoflavones, according to Anderson & Garner (1997).

In rats daidzein conjugates are more bioavailable than genistein conjugates (King 1998). In plasma the maximum concentration of daidzein is approximately double that of genistein after a single oral dose of soy extract (King 1998). The pharmacokinetics of isoflavones are dependent on the form of administration. If isofavone, (like genistein alone or the equivalent dose of its glycone form, as an isoflavone-rich soy extract), is given to the rat, the plasma concentration rises faster in the genistein-treated rats than in the soy-extract-treated rats (King *et al.* 1996). However, eight hours after administration, no differences exist in plasma concentration between the treatment groups (King *et al.* 1996), suggesting that the extent of absorption of genistein may be similar for both the glycone and the aglycone forms.

Genistein and daidzein are available from a diet, and therefore their possible toxic effects have to be considered. Some animal studies of the toxicity of isoflavones exist. Normal

dietary concentrations of genistein and daidzein, present in standard rat chow, are not toxic to rats and have only minimal effects on the reproductive tract (Casanova *et al.* 1999). Neither a single dose of 40 mg/kg nor a cumulative dose of 100 mg/kg of genistein have been shown to exert toxicity in mice (Ek *et al.* 1998), and the intravenous administration of genistein over a period of five days is not toxic to monkeys (Messinger *et al.* 1998).

β-Sitosterol is extracted from such plants as pine or soybean and has estrogenic effects. It increases uterine weight, RNA, DNA and protein concentrations to the same extent as estradiol and more than progesterone (Malini & Vanithakumari 1993). It also increases ovarian weight and has an estrogenic effect on the estrous cycle in rats (Malini & Vanithakumari 1988). On the other hand, β-sitosterol treatment decreases the ability of the testis to produce testosterone in goldfish (MacLatchy & Van Der Kraak 1995), and decreases testicular weight and testosterone concentrations in the blood of male rats (Malini & Vanithakumari 1991).

2.1.2. Hormonal effects of isoflavones

Plant-derived estrogens act as ER agonists or antagonists, depending on the hormonal status of the animal or man. Isoflavonoids at concentrations 100-1000 times higher than that of estradiol-17β have been considered to compete with endogenous mammalian estrogens, to bind ER, and to prevent estrogen-stimulated growth in mammals (for review, see Adlercreutz *et al.* 1995). It is therefore possible that the consumption of a diet rich in plant-derived estrogens could affect endogenic hormone production. The mid-cycle peaks of the luteinizing hormone and the follicle stimulating hormone are suppressed (Cassidy *et al.* 1995) or sometimes unaffected (Lu *et al.* 2000), and in premenopausal women the length of the follicular phase of the menstrual cycle is increased during an isoflavone-rich diet (Cassidy *et al.* 1995; Lu *et al.* 1996). The serum estradiol-17β concentration is unaffected (Cassidy *et al.* 1995; Honoré *et al.* 1997) or decreased (Lu *et al.* 1996; Lu *et al.* 2000), the progesterone level is decreased (Lu *et al.* 1996; Lu *et al.* 2000), and the serum testosterone concentration is unaffected (Honoré *et al.* 1997), or reduced (Strauss *et al.* 1998) by dietary isoflavone supplementation.

One of the most common symptoms of the menopause is hot flushes. The incidence of these is much lower in Malaysia (18%) and China (14%) than in western societies (70-80%) (for review, see Knight & Eden 1995). The urinary levels of isoflavones in Japanese women are 100-1000 times higher than those in omnivorous women (Adlercreutz *et al.* 1992). It has been suggested that this explains the low frequence of menopausal symptoms in the Japanese, although the effects of cultural backgrounds on these symptoms must be taken into account. When soybean flour is added to the diet, the incidence of hot flushes is reduced (Murkies *et al.* 1995). However, dietary wheat flour, which contains few plant-derived estrogens, also reduces hot flushes (Murkies *et al.* 1995). Thus, the precise effect of plant-derived estrogens on hot flushes is still unclear.

Another important menopausal symptom is vaginitis, which is due to epithelial atrophy. When postmenopausal women consume a mixture of soy, linseed and clover, an increase in cell proliferation in the vaginal epithelium occurs (Wilcox *et al.* 1990), indicating estrogenic activity. However, opposite findings also exist. Soybean estrogens have no estrogenic effect on vaginal cytology in postmenopausal macaques (Cline *et al.* 1996) or women (Murkies *et al.* 1995).

Osteoporosis is related to aging and especially to the menopause. After the cessation of ovarian function, women begin to lose their bone mass. The incidence of osteoporosis differs within populations, and according to the World Health Organaization report (1994) the incidence is lower in Asian women than in western women. One of the reasons for this could be the dietary differences between the areas, which are partly related to the consumption of soy products. Soy isoflavones have been shown to attenuate bone loss in perimenopausal women (Alekel *et al.* 2000) and in ovariectomized (OVX) rats (Arjmandi *et al.* 1998a). This may be due to enhanced bone formation rather than to slowed bone resorption (Arjmandi *et al.* 1998b). Although both genistein and daidzein are effective in preventing bone loss, daidzein is the more potent of these two compounds (Picherit *et al.* 2000).

To sum up, genistein and daidzein have estrogenic effects, which may be beneficial in treating menopausal symptoms.

2.1.3. Anticarcinogenic effects of isoflavones

The incidence of breast, endometrial and ovarian cancer is lower in Asia and eastern Europe than in western countries (Rose *et al.* 1986). All these cancers are hormone-dependent. Migrants from Asia who maintain their traditional diet have a decreased risk even when living in western countries (Kolonel 1988), whereas the increased risk of these diseases follows a change towards a westernized diet (Lee *et al.* 1991). An increased soy intake, for example, is associated with reduced breast cancer risk in both pre- and postmenopausal women (Wu *et al.* 1996) and with lowered prostate cancer risk in men (Jacobsen *et al.* 1998; Strom *et al.* 1999). For more references see review by Kurzer & Wu (1997).

The anticarcinogenic effect of isoflavones has been studied widely in animal and *in vitro* models. A soy protein diet, for example, inhibits the growth of prostate adenocarcinoma in mice (Aronson *et al.* 1999; Bylund *et al.* 2000). In rats, three subcutaneous injections of genistein administered neonatally protect against mammary cancer (Lamartiniere *et al.* 1995). On the other hand, in athymic mice, dietary genistein, which produces micromolar plasma concentrations, cannot inhibit the growth of estrogen-independent human breast cancer cells MDA-MB-231, but 10-80 times higher concentrations of genistein do inhibit the growth (Santell *et al.* 2000) and the DNA synthesis (Wang & Kurzer 1997) of these cells in cultures *in vitro*.

Genistein, daidzein (Dixon-Shanies & Shaikh 1999), and biochanin A, a precursor of genistein (Dixon-Shanies & Shaikh 1999; Hsu *et al.* 2000), all inhibit the growth of the human ER positive breast cancer cells MCF-7. However, genistein (Twaddle *et al.* 1999), enterolactone and equol (Welshons *et al.* 1987), a derivative of daidzein, have also been shown to stimulate the growth of MCF-7 cells. The effect of many plant-derived estrogens on the DNA synthesis of MCF-7 cells is biphasic; at low concentrations (0.1-10 μM), genistein, biochanin A and enterolactone stimulate the DNA synthesis, whereas at high concentrations (20-80 μM) their effects are inhibitory (Wang & Kurzer 1997). This suggests that the low concentrations of plant-derived estrogens cause an estrogenic effect on MCF-7 cells, but at high concentrations other mechanisms begin to have an influence. For more references see review by Tham *et al.* (1998).

Because genistein and various other plant-derived estrogens inhibit the growth of both estrogen-dependent and –independent mammary cancer cells, other mechanisms than estrogen antagonism must exist. These mechanisms possibly include tyrosine kinase inhibition (Twaddle *et al.* 1999), the arresting of the cells during the G2/M phase of the cell cycle (Fioravanti *et al.* 1998), the induction of apoptosis (Li *et al.* 1999), the inhibition of angiogenesis (Shao *et al.* 1998), and the inhibition of tumour cell invasion by the down-regulation of matrix metalloproteinase synthesis (Shao *et al.* 1998).

To sum up, plant-derived estrogens have anticarcinogenic effects on both hormone-dependent and hormone–independent cancers *in vitro*, in animal models and also epidemiologically.

2.1.4. Cardiovascular effects of isoflavones

Epidemiologic studies have demonstrated a reduced rate of mortality due to coronary heart disease in Japanese populations consuming a traditional Japanese diet compared to a western diet (Kagan *et al.* 1974). Expatriate Japanese living in the United Kingdom have higher blood pressure and cholesterol levels and lower triglyceride levels than the Japanese still living in Japan (Robinson *et al.* 1995), which suggests that these differences are not of genetic origin but may be due to diet. The Japanese diet is rich in soy products, fish and fibre.

2.1.4.1. Lipid metabolism

In man, the consumption of soy protein has been shown to decrease the serum concentrations of total cholesterol, LDL cholesterol and triglycerides (Anderson *et al.* 1995). The soy protein containing plant-derived estrogens have beneficial effects on serum lipid values, while the soy protein without plant-derived estrogens has no effect in mice (Kirk *et al.* 1998), in rhesus monkeys (Anthony *et al.* 1996), or in man (Crouse *et al.* 1999). However, although plant-derived estrogens without soy protein do not affect serum lipid levels, but they reduce atherosclerotic lesion areas in the aortic arch and lower its cholesterol content at least in rabbits (Yamakoshi *et al.* 2000). The cholesterol-lowering and antiatherosclerotic mechanisms of soy may include reduced absorption of dietary cholesterol (Greaves *et al.* 2000), increased LDL receptor quantity and activity (Baum *et*

al. 1998; Kirk *et al.* 1998), the reduced arterial permeability of LDL, and the reduced arterial concentration and delivery of LDL (Wagner *et al.* 2000). (Table 3)

Oxidized LDL is more prone than unoxidized LDL to remain in vessel wall and to induce atherosclerosis. In man, the intake of genistein and daidzein decreases LDL oxidation (Tikkanen *et al.* 1998). Isoflavones protect against glucose-induced oxidation of human LDL *in vitro* (Vedavanam *et al.* 1999). Both genistein and daidzein have also been shown to protect human umbilical cord endothelial cells and bovine aortic endothelial cells from the atherogenic effect of oxidized LDL (Kapiotis *et al.* 1997). Thus, the antioxidant property of the isoflavones may be important in decreasing the risk of atherosclerosis.

Genistein is a more potent antioxidant than daidzein (Wei *et al.* 1995; Ruiz-Larrea *et al.* 1997). The antioxidant properties of isoflavones are structure related (Wei *et al.* 1995; Ruiz-Larrea *et al.* 1997; Arora *et al.* 1998). The determining factors for isoflavonoid antioxidant activity are the absence of the 2, 3-double bond and the 4-oxo-group on the isoflavone nucleus and the position of the hydroxyl groups, with hydroxyl substitution being of utmost importance at the 4' position, of moderate importance at the 5 position, and of little significance at the 7 position (Arora *et al.* 1998).

To sum up, soy protein improves serum lipid values and inhibits the development of atherosclerosis. These favourable effects may be related to the plant-derived estrogens of soy.

2.1.4.2. Other effects

Although the effects of isoflavones on lipid metabolism are well understood, their other cardioprotective mechanisms have not been widely investigated. However, some studies do exist. For example, isoflavones influence the function of the endothelium. In the atherosclerotic macaque, dietary isoflavones enhance endothelium-dependent relaxation to acetylcholine (ACh) in the coronary arteries (Honoré *et al.* 1997), and treatment with genistein augments endothelium-dependent arterial relaxations in OVX rats (Squadrito *et al.* 2000).

VSMCs contribute to pathological structural changes within the vessel wall by migrating from the media into the intima, and by proliferating and depositing extracellular matrix proteins such as collagen (Dubey *et al.* 1997). Genistein, daidzein, biochanin A and equol inhibit human aortic VSMC proliferation, growth, migration and mitogen-activated protein kinase (MAP) activity (Dubey *et al.* 1999). The order of potency of these plant-derived estrogens is biochanin A > genistein > equol > daidzein (Dubey *et al.* 1999). In menopausal and perimenopausal women, dietary isoflavones improve arterial compliance (Nestel *et al.* 1997). Genistein has also been shown to reduce renal vascular resistance and to act as a diuretic (Gimenez *et al.* 1998), which can be beneficial in regulating blood pressure. (Table 3)

In short, genistein and daidzein affect both the function of the endothelium and the smooth muscle of the vessel wall.

Table 3. Cardiovascular effects of plant-derived estrogens

Population	Plant-derived estrogens	Direction of effect	Reference
In man			
Man	Soy isoflavones	Cholesterol ↓	Anderson *et al.* 1995
Man	Genistein and daidzein	LDL oxidation ↓	Tikkanen *et al.* 1998
Women	Soy isoflavones	Arterial compliance ↑	Nestel *et al.* 1997
In animal			
Rhesus monkey	Soy isoflavones	Cholesterol ↓	Anthony *et al.* 1996
Macaque	Soy isoflavones	Endothelium-dependent relaxations ↑	Honoré *et al.* 1997
Rabbit	Soy isoflavones	Atherosclerotic areas ↓	Yamakoshi *et al.* 2000
Rat	Genistein	Endothelium-dependent relaxations ↑	Squadriato *et al.* 2000
Mouse	Soy isoflavones	Cholesterol ↓	Kirk *et al.* 1998
In vitro			
VSMC culture	Genistein and daidzein	VSMC proliferation ↓	Dubey *et al.* 1999
Kidney perfusion	Genistein	Diuresis ↑	Giménez *et al.* 1998

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Riikka Nevala

2.2. Postmenopausal estrogen replacement therapy (ERT)

Estrogens are female sex hormones produced mainly by the ovaries. They regulate the growth and development of the female sex organs and other tissues related to reproduction. The most important of the estrogens is estradiol-17β. The secretion of estrogens ceases at the age of 45-55 years and the menopause begins. Estrogen replacement therapy (ERT) alleviates menopausal symptoms such as hot flushes and vaginitis. It protects women against many age- and hormone-related diseases such as the osteoporosis and cardiovascular diseases. Long-term use of ERT also improves excercise capacity in postmenopausal women (Redberg *et al.* 2000).

2.2.1. Cardioprotective effects of ERT

Premenopausal women have a lower risk of cardiovascular diseases compared to men of the same age. However, the incidence of coronary heart disease increases when estrogen levels decrease during the menopause. Epidemiological studies have shown an approximately 50% reduction in the risk of coronary incidents in postmenopausal women on ERT compared to non-users (Barret-Connor & Bush 1991). On the other hand, in those populations with low risk of cardiovascular diseases, the use of ERT for 10 years improves life expectancy by only about one month, doubled over 20 years (Moerman *et al.* 2000). However, women who have risk factors of cardiovascular diseases, such as high serum cholesterol and blood pressure, can benefit more from ERT (Moerman *et al.* 2000).

2.2.1.1. Lipid metabolism

For a long time, the beneficial effects of ERT on serum lipid values were considered to be the main reason for the decreased risk of cardiovascular diseases in postmenopausal women. During ERT, total cholesterol (Bongard *et al.* 1998; Chen *et al.* 1998), LDL (Nabulsi *et al.* 1993; Bongard *et al.* 1998; Chen *et al.* 1998; Herrington *et al.* 2000) and triglycerides (Bongard *et al.* 1998; Chen *et al.* 1998) are reduced, and HLD is increased (Nabulsi *et al.* 1993; Herrington *et al.* 2000). On the other hand, in postmenopausal women who use statins to lower cholesterol and to prevent atherosclerosis, ERT has no additional favourable effect on the lipid levels (Os *et al.* 2000).

Increased serum cholesterol and LDL predispose patients to both coronary and peripheral arterial atherosclerosis. Estrogen receptors are more expressed in normal coronary arteries than in atherosclerotic arteries in premenopausal women (Losordo *et al.* 1994), suggesting that the vascular endothelium and smooth muscle cells are targets of estrogen action. This agrees with the finding that ERT for one year or longer reduces the risk of peripheral arterial disease by about one half (Westendorp *et al.* 2000). However, in postmenopausal women with diagnosed coronary disease (Herrington *et al.* 2000) or with increased risk (Angerer *et al.* 2001), ERT combined with progesterone have no effect on the progress of atherosclerosis.

Studies with experimental animals have also demonstrated the protective effect of ERT on atherosclerosis. In OVX rabbits, ERT prevents the development of the disease (Hanke *et al.* 1996), by preventing LDL accumulation in the arterial wall and by decreasing endothelial permeabilty to LDL (Walsh *et al.* 2000). This inhibitory effect of ERT on LDL accumulation may be due to the antioxidant property of estradiol-17β (Walsh *et al.* 1999). The beneficial influence of ERT may be dependent on the function of the endothelium. In OVX rabbits, ERT inhibits cholesterol accumulation if the endothelium is normal, has no effect in reendothelialized areas, and enhances cholesterol accumulation in deendothelialized areas (Holm *et al.* 1999).

Thus, ERT has a beneficial influence on lipid metabolism, and this slows the development of atherosclerosis. However, it is still unclear whether the combining of progesterone with ERT destroys the protective effect of the ERT.

2.2.1.2. Vascular injury

Both ERα and ERβ are expressed in VSMC (Register & Adams 1998). ERT inhibits VSMC proliferation and the increase of vascular media after injury in both ERα (Iafrati *et al.* 1997) and ERβ (Karas *et al.* 1999) deficient mice. This indicates that the protective effect of estradiol-17β requires only one type of functional ER at a time, or that some other still uncharacterized ER is involved. On the other hand, estradiol-17β has been shown to inhibit VSMC growth by stimulating cyclic adenosine 5'-monophosphate (cAMP) synthesis, leading to the formation of adenosine, which regulates growth via A_2 adenosine receptors (Dubey *et al.* 2000). However, the concentration of estradiol-17β needed for activation of

cAMP synthesis is supraphysiological (Christ *et al.* 1999). After injury, the endothelium is also a target of ERT action, because ERT accelerates the recovery of the functional endothelium in OVX rats (Krasinski *et al.* 1997).

2.2.1.3. Coagulation

The use of oral contraceptives has been reported to increase the risk of venous tromboembolism. The risk is relatively high, especially in women using oral contraceptives with a high concentration of ethinylestradiol (for review, see Chasan-Taber & Stampfer 1998). It has recently been shown that ERT plus progesterone also predispose women to tromboembolic events, at least in the case of women with established coronary disease (Grady *et al.* 2000). However, the situation may be totally different in healthy postmenopausal women, because in these subjects, this same medical combination decreases fibrinogen (Nabulsi *et al.* 1993), increases antitrombin III levels (Chen *et al.* 1998), reduces platelet aggregation (Chen *et al.* 1998), and lowers plasma viscosity (Rosenson *et al.* 1998).

2.3. Arterial tone

Arterial tone is an important factor in the regulation of blood pressure. Arterial tone is determined by the interaction of the endothelium and the smooth muscle.

2.3.1. Endothelium

The vascular endothelium is a highly active endocrine organ covering the inner surface of the arteries and veins. In a person weighing 70 kg, the total surface area of the endothelium is about 1100 m^2; it weighs approximately 1800 g, and the total number of cells is in the order of $1x10^{12}$. The endothelium is an important regulator of arterial tone because it secretes various vasodilating (Figure 3) and contracting substances.

2.3.1.1. Vasodilatory factors

Nitric oxide

In 1980 Furchgott and Zawadzki showed that the endothelium must be intact for ACh to induce arterial smooth muscle relaxation. A substance originating from a vessel with an intact endothelium caused relaxation in an arterial ring with a denuded endothelium; it was named “the endothelium-derived relaxing factor” (EDRF). Later the EDRF was confirmed to be nitric oxide (NO) (Ignarro *et al.* 1987; Palmer *et al.* 1987). NO is a gaseous free radical which is synthesized from the amino acid L-arginine by a family of NO syntethases (NOSs). NO relaxes VSMCs by increasing the production of cyclic guanosine 3',5'-monophosphate (cGMP).

A normal endothelium constantly releases small amounts of NO. Extra NO is released in response to physiological stimuli such as increased shear stress and reduced oxygen tension, and to substances such as ACh, bradykinin, histamine, thrombin, ADP, ATP, and the substance P. So far, NO is the most potent vasodilator known (for review, see Umans & Levi 1995). NO also inhibits platelet aggregation, neutrophil adhesion to the endothelium, VSMC proliferation, and adhesion molecule expression. The synthesis of NO is impaired in many diseases e.g. in hypertension, diabetes, hypercholesterolemia and atherosclerosis (for review, see Cannon 1998). In human endothelial cells, NO production is enhanced by estradiol-17β but not by testosterone (Hishikawa *et al.* 1995), and the physiological levels of circulating estradiol-17β elevate basal NO release from endothelial cells (Wellman *et al.* 1996). Pharmacologically, NO synthesis can be blocked by L-arginine analogs such as Nω-Nitro-L-arginine methyl ester (L-NAME).

Prostacyclin

Prostacyclin (PGI_2) is formed from arachidonic acid (AA) by the cyclo-oxygenase enzyme. The endothelial cells are the highest producers of PGI_2, but VMSCs and fibroblast are also able to synthetize PGI_2. PGI_2 is produced in response to shear stress and to substances that stimulate NO formation. The contribution of PGI_2 to vasodilation is less than that of NO. However, PGI_2 inhibits platelet aggregation and promotes fibrinolysis. Estrogens stimulate PGI_2 synthesis in cultured human endothelial cells (Mikkola *et al.* 1996) and in the rat endothelium (Wakasugi *et al.* 1989), whereas testosterone reduces it (Wakasugi *et*

al. 1989). The synthesis of PGI_2 is inhibited by common anti-inflammatory drugs such as acetylsalicylicacid, diclofenac, ibuprofen and tolfenamicacid.

Endothelium-derived hyperpolarizing factor

Endothelium-dependent relaxations and hyperpolarizations can be partially or totally resistant to the inhibitors of cyclo-oxygenase and NO synthetase, suggesting the existence of an additional endothelial relaxing mechanism. These NO- and PGI_2-independent relaxations appear to be without an increase in the intracellular levels of cyclic nucleotides in smooth muscle cells, and the relaxations are antagonized by apamin and ChTX, the inhibitors of Ca^{2+} sensitive K^+ channels (K_{Ca}) (for review, see Félétou & Vanhoutte, 1999). It has been suggested, therefore, that the hyperpolarization of smooth muscle cells caused by the opening of K^+ channels is responsible for these relaxations, and the relaxing agent is called an endothelium-derived hyperpolarizing factor (EDHF). The nature of EDHF was for a long time unclear, but quite recently it has been discovered that EDHF may be an 11,12-epoxyeicosatrienoic acid formed by cytochrome P450 2C from arachidonic acid, at least in the porcine coronary artery (Fisslthaler *et al.* 1999).

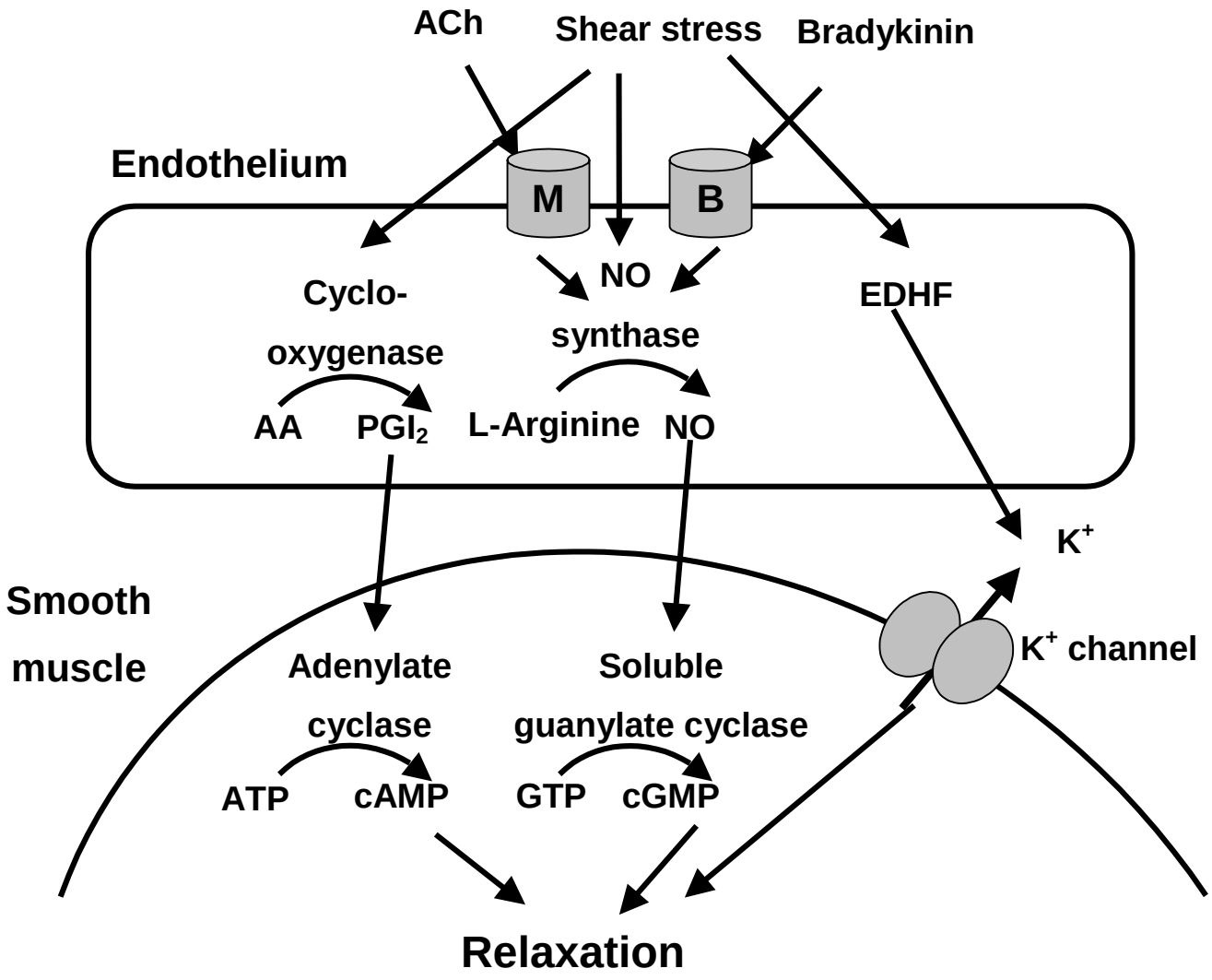

Figure 3. Simplified endothelium-dependent relaxing mechanisms of vascular smooth muscle.

2.3.1.2. Vasocontracting factors

Endothelin-1

Three structurally and pharmacologically separate endothelin isopeptides exist in humans and mammals. These have been named endothelin-1 (ET-1), endothelin-2, and endothelin-3. Endothelial cells can synthesize only ET-1 (for review, see Masaki 1995). ET-1 is synthesized as a proendothelin, which is cleaved to the big endothelin and then, in a reaction, catalyzed by an endothelin-converting enzyme (ECE), and further converted to an active peptide. ET-1 exerts its biological effects by the stimulation of receptors called ET_A and ET_B. ET_A and at lower amount ET_B are present in VSMCs. Their stimulation

induces vasocontraction and mediates the proliferation of VSMCs. ET_B receptors are also present in endothelial cells, where their stimulation is linked to the formation of PGI_2 and NO. However, the most striking property of ET-1 is its long-lasting hypertensive action. It is the most active pressor substance discovered, with a potency 100 times that of angiotensin II (for review, see Masaki 1995).

Angiotensin II

When fluid volume or plasma Na^+ concentration is reduced, the jugstaglomerular cells of the kidney are activated to secrete renin. Renin degrades angiotensinogen to angiotensin I, which is further cleaved to angiotensin II (Ang II) by an angiotensin-converting enzyme (ACE). Ang II contracts VSMC, induces the secretion of aldosterone from the adrenal cortex, and acts as a growth factor in VSMCs (for review, see Stroth & Unger 1999). It binds to its receptors AT_1 and AT_2. The vasoconstrictive and blood pressure increasing effects of Ang II are mainly mediated by AT_1 receptors (for review, see Pueyo & Michel 1997). Ang II also stimulates the release of ET-1 from endothelial cells.

2.3.2. Smooth muscle

2.3.2.1. Potassium channels

Potassium channels (K^+ channels) play an important role in determining smooth muscle excitability and force generation (Figure 4). In the cell membrane of the vascular smooth muscle, four different types of K^+ channels have been identified: voltage-dependent (K_V), Ca^{2+}-activated (K_{Ca}), inward rectifier (K_{IR}), and ATP-sensitive K^+-channels (K_{ATP}) (for review, see Nelson & Quayle, 1995).

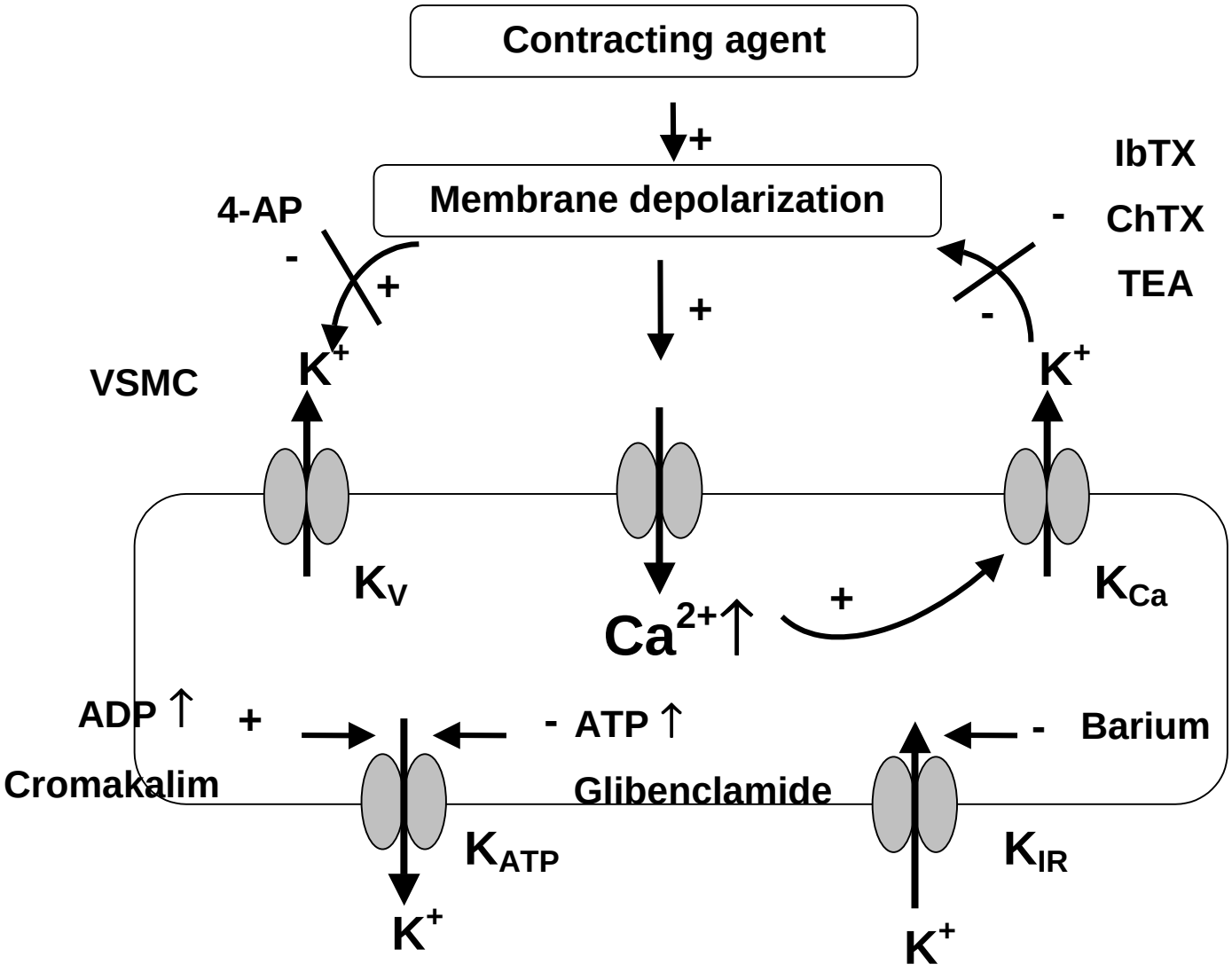

Figure 4. Potassium channels regulating the tone of vascular smooth muscle cells.

Ca^{2+}-activated K^+ channels

K_{Ca}s are of diverse class of K^+ channels that share the common feature of being gated by intracellular Ca^{2+}. Many types of K_{Ca} have been described. They can be divided into three subclasses, based on differences in single-channel conductance, pharmacological properties, and the voltage dependence of channel openings. The high-conductance channels (maxi-channels) (100-300 pS) are usually blocked by ChTX and IbTX, and their gating is voltage-dependent. The intermediate-conductance channels (25-100 pS) are blocked by ChTX, and their gating is not voltage-dependent. Small-conductance channels

(10-20 pS) are typically inhibited by the bee venom toxin, apamin, and their open probability is unaffected by membrane potential. The maxi-channel is probably the most studied of the different types of K_{Ca} channels (for review, see Kaczorowski *et al.* 1996).

The maxi-channels are opened by membrane depolarization and by micromolar concentrations of Ca^{2+}. They are inhibited by tetraethylammonium (TEA) and a family of venom-derived peptides, such as ChTX and IbTX. TEA and ChTX also block other K^+ channels, whereas IbTX is rather selective for maxi-channels. The maxi-channel consists of two dissimilar subunits, α and β. The α subunit is a member of the Ca^{2+}-activated K^+ channel gene family and forms the ion conduction pore. The β subunit is a structurally unique, membrane spanning protein that contributes to channel gating and pharmacology (for review, see Kaczorowski *et al.* 1996). The maxi-channels have been described in a wide range of smooth muscle cells (for review, see Nelson & Quayle 1995). Estradiol-17β-induced relaxation is mediated via maxi-channels in coronaries (White *et al.* 1995). Estradiol-17β binds to the β subunit of this channel and the channel is opened (Valverde *et al.* 1999).

Voltage-dependent K^+ channels

K_V channels belong to the superfamily of voltage-gated channels. The K_V channels open when the membrane potential of the cell is depolarized. They are important regulators of smooth muscle membrane potential. K_V channels are identified in smooth muscle cells of coronary, mesenteric and renal arteries among others (for review, see Nelson & Quayle 1995). 4-Aminopyridine (4-AP) at millimolar concentration is the most selective inhibitor known of K_V channels in vascular smooth muscle. K_V channels are unaffected by glibenclamide, IbTX and ChTX, but TEA inhibits them at high concentrations (for review, see Nelson & Quayle 1995). K_V channels usually show inactivation with sustained depolarization (for review, see Standen & Quayle 1998).

ATP-sensitive K^+ channels

ATP-sensitive K^+ channels (K_{ATP}) are inhibited by physiological concentrations of intracellular ATP and are opened as intracellular ATP falls (for review, see Edwards & Weston, 1993). Under normal physiological conditions the channels are in a closed state (for review, see Edwards & Weston, 1990). K_{ATP} channels exist in different arterial smooth muscle cells (Standen *et al.* 1989). Whole cell K_{ATP} channel currents have been measured

in single smooth muscle cells from pulmonary, coronary and mesenteric arteries (for review, see Nelson & Quayle 1995). K_{ATP} channels are inhibited by extracellular barium and by antidiabetic sulphonylurea drugs such as glibenclamide and glipizide (for review, see Nelson & Quayle 1995). The openers of this channel include minoxidil and cromakalim (for review, see Quast 1993). K_{ATP} channels have been shown to mediate testosterone-induced relaxation in canine coronaries (Chou *et al.* 1996).

Inward rectifier K^+ channels

Inward rectifier K^+ channels (K_{IR}) are present in a variety of excitable and nonexcitable cells including arterial smooth muscle cells. These channels may be preferentially expressed in small rather than large arteries (for review, see Standen & Quayle 1998). The K_{IR} channels are activated by membrane hyperpolarization in contrast to the K_V and K_{Ca} channels, which are activated by membrane depolarization. When the membrane potential is controlled, for instance by a voltage clamp of the cell, the movement of K^+ from extracellular space to intracellular is larger than that from intracellular to extracellular. Extracellular barium in micromolar concentrations is an effective inhibitor of K_{IR} channel (for review, see Nelson & Quayle 1995).

To sum up, K^+ channels seem to mediate estradiol-17β- and testosterone-induced relaxations of the arteries.

2.3.2.2. Calcium channels

In VSMCs two different types of voltage-gated calcium channels (Ca^{2+} channels) exist. Dihydropyridine-sensitive L-type voltage-gated Ca^{2+} channels appear to be dominant in most VSMCs, but T-type voltage-gated Ca^{2+} channels are also present (for review, see Hughes 1995). Voltage-gated Ca^{2+} channels play an important role in regulating vascular tone by membrane potential. Membrane depolarization opens these channels, which leads to vasocontraction, whereas hyperpolarization closes them, causing vasodilation. Ca^{2+} channel blockers are widely used in the treament of hypertension, arrythmias and angina pectoris.

It has been suggested that estradiol-17β relaxes arteries by inhibiting L-type Ca^{2+} channels (Collins *et al.* 1993). In smooth muscle cell line (A7r5), estradiol-17β also inhibits

T-type Ca^{2+} currents (Zhang *et al.* 1994). In isolated smooth muscle cells, this inhibition of Ca^{2+} channels by estradiol-17β is non-genomic, and it reduces myosin light chain phosphorylation and contraction of the smooth muscle (Kitazawa *et al.* 1997). On the other hand, sex hormones also modify the density of Ca^{2+} channels. Long-term female sex hormone deficiency caused by ovariectomy has been reported as increasing and estrogen replacement as decreasing L-type Ca^{2+} channel expression in the rabbit myocardium (Patterson *et al.* 1998). Thus, estradiol-17β regulates both the function and the density of Ca^{2+} channels in muscle cells.

2.3.2.3. Tyrosine phosphorylation

Genistein is a tyrosine kinase inhibitor, while daidzein is inactive in this respect (Akiyama *et al.* 1987). Genistein inhibits tyrosine kinases via interaction with the ATP-binding site (Akiyama *et al.* 1987). Numerous tyrosine kinases have been described, and this superfamily of enzymes has been subdivided into receptor and non-receptor classes (for review, see Courtneidge 1994). Receptor tyrosine kinases are transmembranous proteins possessing intrinsic tyrosine kinase activity, which is regulated by an extracellular ligand, such as a growth factor, whereas non-receptor tyrosine kinases lack the extracellular recognition domain for ligands (for review, see Hughes & Wijetunge 1998). Tyrosine phosphorylation plays a role in such areas as growth, oncogenesis, and smooth muscle contraction.

Tyrosine kinase activity is 500-800 times greater in smooth muscle than in skeletal or cardiac muscles (for review, see Di Salvo *et al.* 1997), indicating that tyrosine kinases are important regulators of the functions of VSMCs. Tyrosine kinase inhibitors have been shown to antagonize vascular contraction in response to a wide range of contractile agents in various arteries *in vitro* (for review, see Hughes & Wijetunge 1998). When $[Ca^{2+}]$ increases inside the smooth muscle cell, it contracts. The early transient increase of $[Ca^{2+}]$ is due to release from the stores and an influx of Ca^{2+}, whereas the lower sustained component of this response is due to Ca^{2+} influx only. Because of its tyrosine kinase inhibiting capacity, genistein antagonizes both these components of Ca^{2+} increase reversibly (for review, see Di Salvo *et al.* 1997). Genistein also regulates the effect of Ca^{2+} on the contractile apparatus (Toma *et al.* 1995).

To sum up, tyrosine kinases regulate the contraction of VSMCs, and the tyrosine kinase inhibitors such as genistein attenuate arterial contractions.

2.4. Blood pressure

High blood pressure is a common health problem in the industrialized countries. One-quarter of the adult population in the USA suffers from hypertension (Burt *et al.* 1995), and in Finland over 10% of the population uses antihypertensive drugs (for review, see Nurminen *et al.* 1998). Hypertension is one of the main risk factors of cardiovascular diseases such as stroke and ischaemic heart diseases (Collins *et al.* 1990; MacMahon *et al.* 1990). Hypertension is also a predisposing factor for left ventricular hypertrophy (LVH), which increases the risk of myocardial infarction, sudden death, cardiac arrhythmias and congestive hearth failure (Frohlich *et al.* 1992). Both clinical and experimental studies have shown that hypertension also has a detrimental effect on the kidneys (Whelton & Klang, 1989).

2.4.1. Protective effects of estrogens on blood pressure and its complications

The higher incidence of hypertension in men and postmenopausal women than in premenopausal women (Kannel *et al.* 1976; for review, see Farhat *et al.* 1996) suggests that female sex hormones in premenopausal women have beneficial vascular effects. The beneficial effects of ERT in postmenopausal women further support a protective role for estrogens against hypertension (Stampfer *et al.* 1991). ERT lowers blood pressure in postmenopausal women (Luotola 1983; Szekacs *et al.* 2000) or leaves it unaffected (Nabulsi *et al.* 1993; Chen *et al.* 1998). Although ERT in some patients lowers blood pressure, it protects neither young (Pedersen *et al.* 1997) nor old (Fung *et al.* 1999) postmenopausal women from stroke. However, LVH is reduced (Lim *et al.* 1999) and left ventricular function is improved (Chen *et al.* 1998) by ERT.

The attenuating effect of estrogen on the development of hypertension has been demonstrated in animal studies. In the normotensive OVX Sprague-Dawley rat (Brosnihan *et al.* 1994), in normal (Hoeg *et al.* 1977; Iams & Wexler 1979; Williams *et al.* 1988) and OVX (Iams & Wexler 1979) female SHRs, in OVX spontaneously hypertensive heart failure rats (Sharkey *et al.* 1999), in OVX trasgenic (mRen2)-27 positive and negative rats

(Brosnihan *et al.* 1997), and in OVX rats with deoxycorticosterone-induced hypertension (Crofton & Share 1997), estrogen treatment attenuates the development of hypertension. On the other hand, in Dahl`s salt-sensitive rats OVX increases the development of hypertension (Hinojosa-Laborde *et al.* 2000). However, when ERT is given to an old postmenopausal spontaneously hypertensive heart failure rat, it has no decreasing effect on already developed hypertension (Sharkey *et al.* 1999). Thus, ERT attenuates the development of hypertension in many rat models, but the beneficial effect disappears if treatment begins after hypertension has already developed.

One important mechanism in lowering blood pressure may be estrogen-induced vasodilation. Estrogen has been shown to relax different arteries in many species - e.g. human (Mügge *et al.* 1993), rabbit (Jiang *et al.* 1991), porcine (Teoh *et al.* 2000) and rat (Otter & Austin 1998) coronary arteries, and rat mesenteric arteries (Naderali *et al.* 1999).

As well as relaxing arterial smooth muscle, estradiol-17β can enhance the relaxations caused by other substances or by physiological stimuli. Both oral ERT (Lieberman *et al.* 1994) and an estradiol-17β infusion (Gilligan *et al.* 1994) improve endothelium-dependent vasodilation in healthy postmenopausal women. ERT combined with progesterone fails to protect women against age-related decline in endothelium-dependent vasodilation (Sorensen *et al.* 1998), suggesting that progesterone may attenuate the beneficial effects of estrogen. However, ERT (Lieberman *et al.* 1994), estradiol-17β infusion (Gilligan *et al.* 1994), and ERT plus progesterone (Sorensen *et al.* 1998) have no effect on endothelium-independent vasodilation in postmenopausal women.

In female SHRs, estrogen treatment enhances the endothelium-dependent relaxations of the aorta (Williams *et al.* 1988). In OVX SHRs, ERT reduces the synthesis of endothelium-derived contracting factors such as $PGH_2/PGF_{2\alpha}$ (Dantas *et al.* 1999) and maintains the NO synthesis (Huang *et al.* 1997). Physiological estradiol-17β levels elevate basal NO release from rat coronary endothelial cells (Wellman *et al.* 1996) and arterial segments (Krasinski *et al.* 1997).

In short, ERT lowers blood pressure in man and attenuates the development of hypertension in animal models. This may be due to the decreased peripheral resistance caused by direct arterial relaxation with estradiol-17β, or by the increased production of

vasodilating substances in the endothelium and the reduced production of vasocontracting ones.

2.4.2. Effect of soy on blood pressure

Some studies on the effect of soy protein and its isoflavones on blood pressure exist. In a clinical trial with normotensive volunteers, the replacement of meat by soy protein did not alter blood pressure (Bursztyn & Van Dias 1985). However, soy protein supplementation, which contains isoflavones, lowers diastolic blood pressure in perimenopausal women (Washburn *et al.* 1999). On the other hand, isoflavones without soy protein have no effect on blood pressure in subjects with high to normal blood pressure levels (Hodgson *et al.* 1999). It is still poorly understood whether soy protein and isoflavones affect the development of hypertension.

3. AIMS OF THE STUDY

Estrogens protect women against cardiovascular diseases by improving serum lipids, by lowering blood pressure and by dilating arteries. Genistein and daidzein are among the most common plant-derived estrogens, which have been shown to lower serum total and LDL cholesterols and to increase serum HDL cholesterol. The mechanisms of action of genistein and daidzein on the cardiovascular system have not been widely investigated.

The aims of the present study were:

1. To compare the effects of estradiol-17β, genistein and daidzein on arterial tone *in vitro* (*Study I*).

2. To clarify the role of potassium channels in estradiol-17β-, genistein- and daidzein-induced arterial relaxation (*Study II*).

3. To examine the effect of gender and ovariectomy on arterial contractions and relaxations (*Study III*).

4. To investigate the effect of a soy-based diet on the development of hypertension in male and female spontaneously hypertensive rats (SHR) (*Study IV*).

5. To evaluate the actions of estradiol-17β and genistein as estrogen receptor agonists and tyrosine kinase inhibitors (*Study V*).

4. MATERIALS AND METHODS

4.1. Experimental animals

Male and non-pregnant female Wistar rats (250-350 g) were purchased from Animal House Arkadia, University of Helsinki. Male, non-pregnant female and OVX female SHRs were purchased form Harlan Sprague Dawley Inc, Indiana, IN, USA. The rats were housed five animals to a cage in a standard experimental animal laboratory, and had free access to tap water. All the rats in *Studies I, II, III,* and *V* were provided with standard laboratory food pellets (R36, Lactamin, Special foderföretaget, Stockholm, Sweden). In *Study IV*, the control group received a standard rat chow (R36, Lactamin, Stockholm, Sweden). One group received a diet produced by adding 20 g of 85.5% casein, (Valio Ltd, Helsinki, Finland) to 80 g of the standard rat chow, and another group received a diet produced by adding 20 g of 90% soy protein (SUPRO 670, Protein Technologies International, St. Louis, Mo, USA) to 80 g of the standard rat chow. All these diets had a final NaCl content of 0.54% and the supplementation period was five weeks. In *Study V*, the following treatments were given subcutaneously once a day for either two days or for two weeks each to the different rat group, as follows: control 96% DMSO 1ml/kg, estradiol-17β 25 μg/kg, genistein 2.5 mg/kg, or genistein 25 mg/kg.

4.2. Arterial responses

4.2.1. Arterial preparations and organ bath solution

The superior mesenteric and renal arteries were carefully excised and cleaned of adherent connective tissue for functional *in vitro* studies (Pörsti *et al.* 1991). One to six 3-mm-long sections of the mesenteric, and one or two 2-mm-long sections of the renal arteries were prepared and placed between stainless steel hooks and mounted in an organ bath chamber in Krebs-Ringer buffer (pH 7.4) of the following composition (mM): NaCl (119.0), $NaHCO_3$ (25.0), glucose (11.1), $CaCl_2x2H_2O$ (1.6), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4x7H_2O$ (1.2), which was then aerated with 96% O_2 and 4% CO_2.

4.2.2. Arterial relaxation and contraction responses

The rings were equilibrated for 1 h at +37°C with a resting tension of 1.0 g for the mesenteric and 0.2 g for the renal arteries. The force of contraction was measured with an isometric force-displacement transducer and registered with a polygraph (FT03 transducer, Model 7P122E Polygraph; Grass Instrument Co., Quincy, MA, USA.). Acetylcholine (ACh)-induced (1 μM) relaxation after noradrenaline (1 μM) precontraction was used to test the presence or the absence of endothelium.

Estradiol-17β-, genistein- and daidzein-induced relaxations

In *Study I*, cumulative relaxations to estradiol-17β, genistein and daidzein were determined after noradrenaline-, potassium chloride- and calcium chloride-induced precontraction in both endothelium-intact and endothelium-denuded mesenteric arterial rings. Each concentration of the relaxing drug was allowed to take its effect for 10 min before the next concentration was administered. Some of the endothelium-intact arterial rings were pretreated with diclofenac or L-NAME or both, in order to study the role of PGI_2 and NO on the relaxations. Pretreatment lasted 15 min. In *Study II*, a single concentration of each compound was administered after noradrenaline precontraction and was allowed to take its effect for 30 min. 15 min before the contraction, the rings were pretreated with different K^+ channel antagonists such as ChTX, IbTX, apamin, 4-AP, barium or glibenglamide, or with an estrogen receptor antagonist, tamoxifen.

Other relaxation responses

The cumulative concentration response curves were studied for ACh and sodium nitroprusside (SNP) (*III, IV, V*) as described by Kähönen *et al.* (1993). In *Study V*, part of the rings were pretreated with diclofenac, L-NAME, TEA or a combination of all these drugs in order to clarify the role of PGI_2, NO and K_{Ca} channels on the ACh-induced relaxations.

Contraction responses

The cumulative concentration-response curves were determined for noradrenaline and potassium chloride (*III, IV, and V*), for calcium chloride in hyperpolarized media *(IV)*, and for ET-1 (*V*). Only a single dose each of Ang I and Ang II was administered in order to avoid tachyphylaxis (*V*).

4.3. Measurement of systolic blood pressure and heart rate

The systolic blood pressure and the heart rate of the pretrained rats were measured at the beginning and at the end of the study (*IV, V*) using a tail-cuff analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA). Before the measurements the rats were warmed for 10-20 min at 30°C to make the pulsations of the tail artery detectable. The values for systolic blood pressure and heart rate were obtained by averaging readings from three consecutive measurements. To minimize stress-induced variations in blood pressure, all measurements were taken by the same person in the same peaceful environment.

4.4. Collection of samples

The rats were kept for 24 h in their metabolic cages. They had free access to chow and tap water. Food and water consumption was determined, and the urine was collected (*V*).

The rats were rendered unconscious with CO_2/O_2 70/30% (AGA, Riihimäki, Finland) and then decapitated. Blood samples were taken and placed in chilled tubes and centrifuged at +4°C (*IV, V*). The heart was excised, the large blood vessels, the atria and the free wall of the right ventricle were dissected, and the left ventricular was weighed (*IV, V*). The kidneys, uteri (*IV, V*), testicles and ovaries *(IV)* were excised, washed with ice-cold saline and weighed. Organ-weight-to-body-weight ratios were calculated to describe the hypertrophy or atrophy of these organs.

4.5. Histology

Tissue samples from the superior mesenteric artery, the right renal artery and the uterine horns were prepared and fixed for 24-48 h with 10% formaldehyde. The samples were dehydrated and embedded in paraffin using the standard protocol. Cross-sections (5 μm thick) of the arteries and uteri were deparaffinized, hydrated, and stained with Masson's trichrome, or with hematoxylin and eosin. The slides were examined in a blinded fashion (*V*).

4.6. Biochemical determinations

4.6.1. Serum cholesterol, LDL, HDL and triglyceride concentrations

Serum cholesterol and triglyceride concentrations were measured with enzymatic colorimetric tests (Boehringer Mannheim GmbH, Mannheim, Germany), and serum HDL concentrations with a homogeneous enzymatic colorimetric test (Boehringer Mannheim GmbH, Mannheim, Germany). Serum LDL concentrations were estimated with the Friedewald formula.

4.6.2. Serum estradiol-17β and testosterone concentrations

Serum estradiol-17β concentrations were measured with a competetive radioimmunoassay (RIA) (Orion Diagnostica, Turku, Finland). Before RIA, the samples were extracted with diethylether, the organic phase was evaporated and the dried residue was dissolved with estradiol zero serum. Serum testosterone concentrations were measured with an automated direct competitive chemiluminoimmunoassay (CIA) (Chiron Diagnostics Corporation, Emeryville, CA, USA).

4.6.3. Urinary creatinine and electrolyte excretions

Urine creatinine was analyzed by the Jaffe method (Bartels *et al.* 1972) (BM/Hitachi 917 analyzer, Boehringer Mannheim, Germany/Hitachi Ltd, Tokyo, Japan) without deproteinization. Urine sodium and potassium were determined by a flame photometer using an ion-selective electrode compensator (human serum pool, IL model 943, Instrumentarium Laboratory, Milan, Italy). Urine calcium was determined by the method described by Cali *et al.* (1973).

4.6.4. Aortic nitric oxide synthase expression

Aorta tissues were homogenized, using an Ultra-Turrax homogenizer, in five volumes of boiling lysis buffer (1% sodium docecyl sulfate (SDS), 1.0 mM Na_3VO_4, 10 mM Tris pH 7.4) (Sigma, St. Louis, MO, USA). The protein content of the supernatants was measured according to the method of Lowry *et al.* (1951). Equal amounts of protein (30 μg) were

resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to the nitrocellulose membranes (Hybond-C Extra, Amersham, Buckinghamshire, UK) (Laemli 1970). The membranes were incubated with the mouse monoclonal anti-eNOS IgG_1 (1:2500,Transduction Laboratories, Lexington, KY, USA), followed by incubation with the horseradish peroxidase-coupled anti-mouse IgG_1 (1:1000, Zymed Laboratories, San Francisco, CA, USA). The bound antibodies were detected using an ECL (enhanced chemiluminescence) reagent (Amersham). Homogenized rat aortas and prestained molecular marker proteins (Bio-Rad Laboratories, Hercules, CA, USA) were used as a positive control. Each band was quantified with computer programmes (GeneSnap and GeneTools, Synoptics, Cambridge, UK).

4.6.5. Vascular smooth muscle tyrosine phosphorylation

To study tyrosine phosphorylation, the mesenteric arterial (*II*) or aortic (*V*) rings were homogenized, using an Ultra-Turrax homogenizer, in five volumes of boiling lysis buffer (10 mM Tris, pH 7.4, 1% sodium docecyl sulfate (SDS), 1.0 mM Na_3VO_4, 50 mM NaF). The protein content of the supernatants was measured according to the method of Lowry *et al.* (1951). The supernatants were boiled in reducing Laemmli sample buffer, and 10 µg samples were separated in 4-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunodetection was performed using an anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology, New York, NY, USA), biotinylated anti-mouse immunoglobulins (Dako A/S, Glostrup, Denmark) and streptavidin-biotin horseradish-peroxidase-conjugated secondary antibodies (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK), followed by detection by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

4.7. Cell cultures

Rat aortic smooth muscle cells were isolated by an explant method (Ross 1971) from the thoracic aortas of male Wistar rats. The smooth muscle cells were cultured in Dulbecco`s Modified Eagle`s Medium/Nutrient Mixture F-12 (DMEM/F12, 1:1) supplemented with 10% fetal bovine serum, HEPES (15 mM), and antibiotics. The cells (passages 4-6) were pretreated with genistein (10-100µM), daidzein (10-100µM) or a control vehicle, for 15-30 min before the addition of orthovanadate (10-100µM). After 10-15 min of incubation with

orthovanadate, the cells were lysed with a Triton lysis buffer (50 mM TRIS pH 7.4,10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 1mM Na_3VO_4, and protease inihibitors). The protein concentrations of the supernatants were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories). SDS-PAGE and immunodetection were performed as described above.

4.8. Compounds

The following compounds were used: acetylcholine chloride, angiotensin I acetate, angiotensin II acetate, 4-aminopyridine, apamin, charybdotoxin, daidzein (purity 98%), diclofenac, endothelin-1, estradiol-17β (purity 98%), genistein (purity 98%), iberiotoxin, Nω-Nitro-L-arginine methyl ester, noradrenaline bitartrate, sodium orthovanadate, tetraethylammonium acetate (Sigma Chemical Co, St. Louis, MO, USA), barium chloride (E. Merck AG, Darmstad, Germany), β-sitosterol (Kaukas Ltd, Chemical Mill, Lappeenranta, Finland), glibenclamide (Leiras Ltd, Turku, Finland), sodium nitroprusside (F. Hoffmann La Roche Ltd, Basel, Switzerland) and tamoxifen citrate (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA). The estradiol-17β (*I, II*) genistein (*I, II*), daidzein (*I, II*), β-sitosterol and tamoxifen were dissolved in ethanol. The 4-Aminopyridine, barium chloride, estradiol-17β (*V*), genistein (*V*), glibenclamide and tetraethylammonium were dissolved in DMSO (96%). The other compounds were dissolved in water. The solutions were prepared just before use and protected from light.

4.9. Statistical analysis

The results are expressed as means ± S.E.M. The sensitivity of the artery to the cumulative relaxation response is presented as pD_2 values, which are calculated as the negative log of the dose required to produce the half-maximal response (*I, IV, V*). The results of cumulative concentration response curves were analyzed using two-way analysis of variance (MANOVA) with repeated measurements. Duncan`s test (*III, IV*) and Newman-Keuls` test (*I, V*) were used for pairwise comparisons between the treatment groups. Other data were analyzed with one-way analysis of variance. Newman-Keuls` test (*II*) and a Student`s t-test (*V*) were used for the comparison of the means. $P < 0.05$ was considered significant.

4.10. Ethics

The studies were approved by by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki.

5. RESULTS

5.1. Arterial tone

5.1.1. Effects of estradiol-17β, genistein, daidzein and β-sitosterol on arterial tone *in vitro*

In endothelium-intact female mesenteric arterial rings precontracted with either noradrenaline (1 μM), potassium chloride (60 mM), or calcium chloride (1 mM), estradiol-17β (1-100 μM), genistein (1-100 μM) and daidzein (1-100 μM) induced a cumulative dose-dependent relaxation response compared to an ethanol control in female rats. These relaxation responses were also tested with male mesenteric arterial rings, and no statistical difference was found compared to the rings from female rats. β-Sitosterol (1-100 μM) only relaxed noradrenaline precontracted endothelium-intact rings, but the relaxation was not statistically significant. In potassium chloride and calcium chloride precontracted rings, estradiol-17β was the most potent relaxant, and genistein was more potent than daidzein. Pretreatments with diclofenac, an inhibitor of PGI_2-production, or L-NAME, an inhibitor of NO-synthesis, did not alter these relaxations. No differences existed between the endothelium-intact and –denuded rings (*I*).

Estradiol-17β-induced (10 μM) relaxation was inhibited by IbTX (30%) but not by ChTX, both of which are inhibitors of large conductance K_{Ca}-channels. Apamin, an inhibitor of small conductance K_{Ca}-channels, attenuated estradiol-17β-induced relaxation by 45%. Genistein-induced (10 μM) relaxation was also significantly inhibited by IbTX (50%) and ChTX (60%), but not by apamin. The relaxation response of daidzein (10 μM) was reduced by IbTX (50%), ChTX (60%) and apamin (50%) (*II*).

4-AP, an inhibitor of K_V-channels, had no effect on estradiol-17β-, genistein- or daidzein-induced relaxation responses. Nor did glibenclamide, an inhibitor of K_{ATP}-channels, barium, an inhibitor of K_{IR}-channels, or TEA, an unspecific inhibitor of K_{Ca}-channels, alter the relaxation responses (*II*).

To sum up, the relaxations induced by estradiol-17β, genistein and daidzein were endothelium- and gender-independent, and the relaxations were mediated, at least partly, via K_{Ca}-channels.

5.1.2. Effects of gender, ovariectomy, low- and high-dose genistein, and estradiol-17β treatments on arterial tone

Mesenteric artery

In normotensive Wistar rats, male gender augmented the mesenteric arterial contractions to noradrenaline (10 nM-10 μM) by approximately 50% and to potassium chloride (20-125 mM) by 30% compared to female rats with intact ovaries. The OVX Wistar female rats had 25% higher noradrenaline-induced and 20% higher potassium chloride-induced contractions than normal female rats (*III*). In both male and female SHRs, the soy protein diet had no effect on noradrenaline- (1 nM-100 μM), potassium chloride- or calcium chloride-induced (10 μM-10 mM) contractions (*IV*).

The endothelium-dependent relaxations to ACh (1 nM-10 μM) were unaffected by gender and OVX, whereas the endothelium–independent relaxations to SNP (1 nM-10 μM) were enhanced in normal female Wistar rats compared to the males (*III*). OVX shifted the dose-response curve to SNP to the right, in the direction of that of the males (*III*). In the male and female SHRs, the soy protein diet had no effect on ACh- or SNP-induced relaxations (*IV*). In OVX SHRs, neither the two-day low-dose (2.5 mg/kg/d) nor the high-dose genistein (25 mg/kg/d), nor the estradiol-17β (25 μg/kg/d) treatments influenced the ACh- or SNP-induced maximum relaxations *ex vivo*. When diclofenac, L-NAME and TEA were added *in vitro* as pretreatments, the maximum ACh relaxations did not differ between the genistein and estradiol-17β groups. After the two-week genistein or estradiol-17β treatments, ACh- and SNP-induced relaxations were similar to those seen after the two-day treatments (*V*).

To sum up, male gender and OVX in Wistar rats increased noradrenaline- and potassium chloride-induced contractions. Male gender and OVX decreased the SNP-induced relaxations, while neither the soy protein supplementation, nor the low- or high-dose genistein, nor the estradiol-17β treatments had any effect on endothelium-dependent or -independent relaxations in the mesenteric artery (Table 4).

Renal artery

Renal artery contractions were attenuated by the two days of low-dose genistein treatment as follows: Ang II (1 μM) (46%), noradrenaline (42%), potassium chloride (36%), and ET-1 (0.1 nM-33 nM) (34%). Only the Ang II-induced contractions were reduced by the two-week treatment with estradiol-17β (38%) and with the low-dose of genistein (31%) compared to the controls. No differences existed in Ang I-induced (1 μM) contractions between the treatment groups after the two-day or two-week interventions (*V*).

The relaxations induced by ACh (1nM-1 μM) and SNP (0.1 nM-1 μM) were unaffected by the genistein or estradiol-17β treatments. SNP-induced dose-response curves shifted to the left in all the groups after the two-week treatments compared to the two-day treatments. At the end of two weeks the controls were the most sensitive to SNP relaxation (*V*).

In short, the two-day low-dose genistein treatment of the OVX SHRs attenuated renal arterial contractions *ex vivo*, whereas the endothelium-dependent and -independent relaxations remained unaffected (Table 4).

Table 4. Summary of effects of male gender, OVX, soy protein diet, and genistein and estradiol-17β treatments on arterial function in rats. NA noradrenaline; KCl potassium chloride; ACh acetylcholine; SNP sodium nitroprusside; ET-1 endothelin-1; Ang II angiotensin II.

Mesenteric arteries	**NA**	**KCl**	**ACh**	**SNP**
Normotensive rats				
Male gender	↑	↑	↔	↓
OVX	↑	↑	↔	↓
SHRs				
Soy protein diet	↔	↔	↔	↔
OVX SHRs				
Low-dose genistein			↔	↔
High-dose genistein			↔	↔
Estradiol-17β			↔	↔

Renal arteries	**NA**	**KCl**	**ACh**	**SNP**	**ET-1**	**Ang II**
Two-day treatment						
Low-dose genistein	↓	↓	↔	↔	↓	↓
High-dose genistein	↔	↔	↔	↔	↔	↔
Estradiol-17β	↔	↔	↔	↔	↔	↔
Two-week treatment						
Low-dose genistein	↔	↔	↔	↔	↔	↓
High-dose genistein	↔	↔	↔	↔	↔	↔
Estradiol-17β	↔	↔	↔	↔	↔	↓

↑ increased

↓ decreased

↔ no effect

5.2. Blood pressure

Systolic blood pressure was 15 mmHg lower in female and 19 mmHg lower in male SHRs on a soy protein diet compared to female and male SHRs on a casein diet. There were no differences in blood pressure values between the female and the male SHRs (*IV*). The two-week treatments with estradiol-17β (25 μg/kg/d), low-dose genistein (2.5 mg/kg/d) or high-dose genistein (25 mg/kg/d) did not significantly alter blood pressure compared to the OVX SHR controls (*V*).

5.3. Body and organ weights

The casein or soy protein supplementation did not alter the body weights in any of the different diet groups in the SHRs. The male rats weighed about 50% more than the respective females *(IV)*. In OVX SHRs, the two-week treatment with estradiol-17β and high-dose genistein lowered body weights compared to the controls (*V*).

Both soy and casein supplementation in the diet induced a significant renal hypertrophy in both female and male SHRs compared to the SHRs on the control diet. Neither the soy nor the casein supplementation altered uterial, ovarial or testical weights (*IV*). In the OVX SHRs, the two-day treatment with estradiol-17β and high-dose genistein induced hypertrophy in the uteri (*V*), as did the two-week treatment with both low- and high-dose genistein and with estradiol-17β.

5.4. Tissue morphology

Both the two-day and the two-week estradiol-17β treatments, as well as the two-week high-dose genistein treatment, all caused estrogenic changes in uterine morphology (*V*).

Neither the two-day nor the two-week treatment with estradiol-17β, nor the low- or high-dose genistein altered the morphology of the renal and mesenteric arteries in the OVX SHRs. Every sample had normal intimal, medial and adventitial morphology (*V*).

5.5. Biochemical determinations

5.5.1. Serum cholesterol, LDL, HDL, and triglyceride concentrations

The five-week soy protein supplementation reduced serum total cholesterol levels in both the female rats (20%) and the male rats (10%), and HDL-levels in the female rats (20%). Serum total cholesterol, HDL and triglycerides were all 30-45% higher in the female rats compared to the males. The different diets did not affect triglyceride levels. Serum LDL-concentrations were below the detection limit in all the groups (*IV*).

5.5.2. Serum estradiol-17β and testosterone concentrations

The soy protein supplementation reduced serum estradiol-17β concentration by 60% compared to the control diet. The serum testosterone concentrations were unaffected by the soy or the casein diets in SHRs (*IV*).

5.5.3. Urinary creatinine and electrolyte excretions

The daily urine sodium and calcium excretions decreased, (17% and 40% respectively), in the high-dose genistein animals compared to the control rats, while no differences appeared between the groups in the daily urine potassium excretion. Creatinine excretion was also similar in all the OVX SHR groups (*V*).

5.5.4. Aortic nitric oxide synthase expression

Neither the two-day nor the two-week treatments with estradiol-17β, low- or high-dose genistein affected the amount of eNOS protein measured by western blotting in the aortic tissue of the OVX SHRs (*V*).

5.5.5. Vascular smooth muscle tyrosine phosphorylation

Orthovanadate (100 μM) increased tyrosine phosphorylation in the mesenteric arterial rings. Genistein pretreatment for 15 min at a concentration of 10 μM slightly reduced phosphorylation, and at a 10 times higher concentration decreased it clearly. Both daidzein

10 μM and 100 μM pretreatments reduced phosphorylation slightly, but less than genistein 100 μM (*II*).

In the VSMC culture, orthovanadate increased tyrosine phosphorylation. Neither a low concentration of genistein (10 μM) nor a daidzein pretreatment modified phosphorylation by orthovanadate. The pretreatment with a higher concentration of genistein (100 μM) decreased the orthovanadate-induced phosphorylation. Daidzein was clearly less effective (*II*).

The two-day low-dose genistein treatment decreased tyrosine phosphorylation in the aortic rings. The two-week high-dose genistein treatment did not alter phosphorylation compared to the controls. The inhibitory effect of the low-dose genistein treatment on tyrosine phosphorylation disappeared when the two-week treatment was completed (*V*).

In conclusion, genistein decreased tyrosine phosphorylation in the mesenteric and aortic rings *in vitro* and *ex vivo*, as well as in the cultured VSMCs.

6. DISCUSSION

The protective role of estradiol-17β in cardiovascular diseases is well known, while the effects of genistein and daidzein and their possible mechanisms have not been so widely studied. In the present study, the effects of the plant-derived estrogens genistein and daidzein on arterial tone and blood pressure were investigated and compared to those of estradiol-17β. Normotensive male and female rats (*Studies I, II, and III*), ovariectomized normotensive female rats (*Study III*), male and female spontaneously hypertensive rats (*Study IV*), and ovariectomized spontaneously hypertensive rats (*Study V*) were used. The study evaluated in particular the role of gender, endothelium, potassium channels, estrogen receptors and tyrosine phosphorylation in genistein- and daidzein-induced arterial responses.

6.1. Methodology

The superior mesenteric artery of rats was used as a model of the resistance artery in all the experiments. The mesenteric artery is suitable for organ bath studies because it produces a stable precontraction and its diameter is about 1-1.5 mm, which allows its preparation without a light microscope. The renal artery was also studied in one experiment, because it regulates the perfusion of the kidney and in this way participates in the control of blood pressure.

Plasma genistein and daidzein concentrations reach micromolar level after a soy-based meal in man (King & Bursill 1998) and in rat (King *et al.* 1996; King 1998). Thus, the concentrations of genistein and daidzein used in the present study to induce arterial relaxations *in vitro*, are physiologically achievable. On the contrary, the normal estradiol-17β concentration in premenopausal woman is within the nanomolar range. In the present study the concentrations of estradiol-17β, genistein and daidzein needed to induce relaxation *in vitro* were micromolar. This phenomenon - that the effective *in vitro* concentration is far from physiological concentration - is often described in connection with estradiol-17β (Jiang *et al.* 1991; Mügge *et al.* 1993).

Because genistein and daidzein are ER agonists (Miksicek 1993), their hormonal effects were investigated. The serum estradiol-17β and testosterone concentrations of rats as well as the weights of the uteri, ovaries, and testes were measured, and the morphologis of the uteri were investigated.

Wistar rats are normotensive rats, and are resistant to cardiovascular diseases. The mesenteric arteries of Wistar rats served as a model of an artery with normal functional structure, whereas the arteries of adult SHRs often have alterations in their structure and function caused by hypertension. A number of the Wistar rats and SHRs were ovariectomized in order to arrest the production of estrogens and to induce a "postmenopausal" state in the rats. A lack of estrogens causes the same state in rats as in humans osteoporosis for instance, (Arjmandi *et al.* 1998a; Arjmandi *et al.* 1998b), and elevated blood pressure (Hinojosa-Laborde *et al.* 2000).

SHRs were studied because the SHR is a commonly used, and regarded as a good animal model of human essential hypertension. The SHR is an inbred strain, which develops hypertension and its complications with increasing age. Systolic blood pressure was measured by the tail cuff method, which has been reported to show good correlation to the recordings using intra-arterial catheters in rats (Bunag & Butterfield 1982). The main limitations are that diastolic blood pressure cannot be obtained and that the measurement cannot be carried out in freely moving animals. The variability of blood pressure values is also large, but this can be partly eliminated if only one person handles the animals and carries out the measurements in a peaceful environment.

6.2. Endothelium- and estrogen-receptor-independent relaxations of estradiol-17β, genistein and daidzein

Estradiol-17β, genistein and daidzein relaxed rat mesenteric arterial rings endothelium-independently. It has been demonstrated that endothelium is not necessary for either female or male sex-hormone-induced relaxations *in vitro*. Estradiol-17β (Jiang *et al.* 1991), progesterone (Jiang *et al.* 1992) and testosterone (Yue *et al.* 1995) all relax arterial smooth muscle endothelium-independently. Genistein also relaxes rabbit coronaries endothelium-independently (Figtree *et al.* 2000). Although the relaxations caused by estradiol-17β, genistein and daidzein are not mediated via the endothelium *in vitro*,

endothelial cells do contain ERs (Kim-Schulze *et al.* 1996). Estradiol-17β increases the synthesis of PGI_2 (Mikkola *et al.* 1996) and NO (Hayashi *et al.* 1995) ER-dependently, suggesting that the endothelium is a target for sex hormone action in some situations.

Tamoxifen, an antagonist of ER, did not inhibit estradiol-17β- genistein- or daidzein-induced relaxations. All these relaxations began about two to four minutes after the compound had been added to the organ bath chamber, which further supports the theory that acute hormone-induced relaxations *in vitro* are not mediated via ERs. Other investigators have achieved similar results: estradiol-17β (Jiang *et al.* 1991; Shaw *et al.* 2000), genistein (Figtree *et al.* 2000; Mishra *et al.* 2000), and daidzein (Mishra *et al.* 2000) have been shown to induce vasodilation via a non-genomic mechanism. Moreover, estradiol-17α, which is a steroid, devoid of genomic estrogenic effects, is also able to relax arteries (Salas *et al.* 1994). All these results suggest that ERs are not involved in estradiol-17β-, genistein- and daidzein-induced relaxations *in vitro*. However, both ERα and ERβ are expressed at least in the coronary arteries and in cultured aortic smooth muscle cells (Register & Adams 1998), suggesting that the VSMCs are targets of estrogen action. Estradiol-17β inhibits the proliferation of VSMCs ER-dependently (Cathapermal *et al.* 1998), and the blocking of ERs induces VSMC proliferation (Lavigne *et al.* 1999). Thus, although ERs are not involved in the acute vasodilatory effect of estradiol-17β, genistein and daidzein, ERs are of importance in regulating the function of VSMC.

6.3. Role of potassium channels in estradiol-17β-, genistein- and daidzein-induced relaxations

Estradiol-17β-, genistein- and daidzein-induced relaxations were antagonized by IbTX, which is an inhibitor of large conductance K_{Ca} channels. This accords with the finding that in the rabbit coronary artery, estradiol-17β-induced relaxation is mediated via the K_{Ca} channels (White *et al.* 1995). The supposed mechanism of the interaction of estradiol-17β and the K_{Ca} channel is that estradiol-17β binds directly to the regulatory (β) subunit of the K_{Ca} channel and activates it (Valverde *et al.* 1999). This leads to the hyperpolarization and relaxation of smooth muscle. Whether genistein and daidzein bind similarly to K_{Ca} channels is still unclear. However, genistein, but not daidzein, has been shown to increase K_{Ca} channel currents in vascular smooth muscle (Xiong *et al.* 1995), and in trabecular

meshwork (Stumpff *et al.* 1999) cells. Thus, it is possible that genistein acts in the same way as estradiol-17β in activating K_{Ca} channels.

In the present study, estradiol-17β-induced relaxation was reduced by IbTX, but not by ChTX, or by TEA, all of which antagonize large conductance K_{Ca}.channels. TEA, however, is an unspecific K^+ channel blocker. Depending on the concentration used, it inhibits K_{Ca}, K_{ATP}, and K_V channels (for review, see Nelson & Quayle 1995). TEA binds with moderate affinity to the external pore of the K_{Ca} channel (for review, see Kaczorowski *et al*. 1996). ChTX is a more selective inhibitor of this channel, but IbTX is even better (for review, see Kaczorowski *et al*. 1996). The dissociation rate of IbTX from the pore of the K_{Ca} channel is much slower than for ChTX (Candia *et al.* 1992). To sum up, IbTX is the most selective inhibitor of K_{Ca} channels, and therefore it is logical that it was the most potent inhibitor of the relaxations that were studied.

Because IbTX inhibited the relaxation responses of estradiol-17β, genistein and daidzein, these relaxations seem to be mediated, at least partly, via K_{Ca} channels. However, in tracheal smooth muscle, IbTX decreases the relaxation response to salbutamol, but this inhibitory effect is totally reversed by nifedipine, (Huang *et al.* 1993), which is an antagonist of L-type voltage-dependent Ca^{2+} channels. This suggests that IbTX causes membrane depolarisation, which in turn activates voltage-dependent Ca^{2+} channels, and the influx of Ca^{2+} via these channels increases the arterial contraction level, which overcomes relaxation. This indicates that the antagonism produced by this toxin is functional and not at the level of the gating on K_{Ca} channels. On the other hand, it is typical for K^+ channel openers, as for levcromakalim, that it cannot relax VSMCs in a K^+-rich medium (Cook *et al.* 1995). In *Study I*, estradiol-17β, genistein and daidzein all relaxed mesenteric arterial rings precontracted by potassium chloride 60 mM, indicating that these substances are not pure K^+ channel openers. Therefore, the possibility that the inhibitory effect of IbTX on the arterial relaxations induced by estradiol-17β, genistein and daidzein may be of a functional nature, cannot totally be excluded.

Glibenclamide, a specific antagonist of the K_{ATP} channel, had no effect on the relaxations induced by estradiol-17β, genistein or daidzein, indicating that K_{ATP} channels do not play an important role in these responses. In porcine coronary arteries, glibenclamide does not

affect estradiol-17β-induced relaxations, either (White *et al.* 1995). On the other hand, K_{ATP} channels have been shown to mediate testosterone-induced relaxation responses *in vivo* in coronaries (Chou *et al.* 1996). Interestingly, it has been reported that in the coronary artery preparation *in vitro*, glibenclamide does not reduce the relaxation induced by testosterone (Yue *et al.* 1995). It is therefore possible that K_{ATP} channel inhibitors could alter estradiol-17β-, genistein- and daidzein-induced relaxations in *in vivo* models. On the other hand, genistein inhibits, and does not activate, K_{ATP} channels in the rabbit portal vein smooth muscle *in vitro* (Ogata *et al.* 1997), which indicates differences between arterial and venous smooth muscles in the function of K_{ATP} channels.

Clearly, estradiol-17β, genistein and daidzein are not pure K^+ channel openers. Therefore, other relaxing mechanisms are likely to exist. It has been suggested that estradiol-17β relaxes arteries by blocking Ca^{2+} channels (Collins *et al.* 1993). In isolated VSMCs, pharmacological (0.1-10 μM) concentrations of estradiol-17β inhibit L-type Ca^{2+} channel currents (Kitazawa *et al.* 1997). Genistein (Figtree *et al.* 2000) and some estrogenic pollutants (Ruehlmann *et al.* 1998) have been shown to relax VSMCs by blocking L-type Ca^{2+} channels. Genistein also reduces voltage dependent Ca^{2+} currents in VSMCs (Wijetunge *et al.* 1992), and daidzein is almost as effective as genistein in inhibiting L-type Ca^{2+} channels in ventricular cells (Yokoshiki *et al.* 1996). To sum up, it is possible that part of the relaxation caused by estradiol-17β, genistein and daidzein is due to the inhibition of L-type Ca^{2+} channels.

6.4. Role of tyrosine kinase inhibition of genistein in arterial responses

Apart from being an estrogen agonist, genistein is also a tyrosine kinase inhibitor (Akiyama *et al.* 1987), whereas daidzein is inactive in this respect. Tyrosine kinase inhibitors attenuate arterial contractions to many substances (for review, see Hughes & Wijetunge 1998). Sodium orthovanadate, which increases tyrosine phosphorylation (Laniyonu *et al.* 1994), on the other hand, induces contraction in VSMCs (Shimada *et al.* 1986). In *Studies I* and *II,* genistein was a slightly more effective relaxant than daidzein. In *Study II*, genistein inhibited more tyrosine phosphorylation than daidzein, both in the mesenteric arterial rings and in the cultured aortic VSMCs. The greater relaxing capacity of genistein than daidzein suggests that part of the relaxing property of genistein may be due to the decreased level of tyrosine phosphorylation in VSMCs, but this may not be the only mechanism.

In *Study V*, the two-day low-dose genistein treatment attenuated renal arterial contractility to all the contracting compounds tested in OVX SHRs. Tyrosine phosphorylation was reduced in aortic smooth muscle by the two-day genistein treatment, whereas neither the two-day treatment of estradiol-17β nor the high-dose genistein treatments reduced the phosphorylation level. Other investigators have shown that genistein reduces renal vascular resistance (Giménez *et al.* 1998). Genistein and other tyrosine kinase inhibitors antagonize the increase of [Ca^{2+}] inside the VSMCs reversibly (for review, see Di Salvo *et al.* 1997). They also regulate the effect of Ca^{2+} on the contractile apparatus of VSMCs (Toma *et al.* 1995). Because of these effects both the depolarization and the contraction of VSMCs are antagonized. Thus, genistein inhibits tyrosine kinase and in this way modifies renal arterial contractility.

It is interesting that the renal arterial contractions and aortic smooth muscle tyrosine phosphorylation were unaffected by the two-day high-dose (25 mg/kg) genistein treatment. The tyrosine kinase inhibitor activity of genistein is evident at high doses (up to 1mg/kg) (Akiyama *et al.* 1987). Our results suggest that at very high doses genistein loses its specificity to inhibit tyrosine kinase. The decreasing effect of the low-dose genistein treatment on renal arterial contractions and tyrosine phosphorylation disappeared after the two-week treatment. It is possible that tyrosine phosphorylation is compensatorily increased after the drop of the basal level during the first days of a genistein treatment.

6.5. Effect of male gender and ovariectomy on mesenteric arterial responses *ex vivo*

Male gender and OVX increased mesenteric arterial contractility compared to normal female rats in both a receptor and a depolarization mediated way. Other researchers have shown that estradiol-17β inhibits VSMC proliferation ER-dependently in female rats (Espinosa *et al.* 1996) and in pigs (Vargas *et al.* 1993). Estradiol-17β also activates a cAMP-adenosine pathway in VSMCs, which inhibits growth (Dubey *et al.* 2000). Thus, the lack of estrogens in male and OVX rats can enhance arterial contractility by increasing VSMC mass, which increases the force of the contractions.

Noradrenaline activates adrenergic α_1 and α_2 receptors. The absence or presence of estrogen modifies the receptor density of VSMCs. ERT reduces the density of α_2 receptors

in OVX rat arteries (Gisclard *et al.* 1987) and suppresses α_1 receptor expression in OVX rat VSMCs (Zhang & Davidge 1999). However, testosterone stimulates the expression of α_1 receptors in VSMCs (Philippe *et al.* 1991). Therefore, the increased contractions to noradrenaline in male and OVX female rats in the present study may be the result of altered α_1 and α_2 receptor densities.

One possible explanation for increased depolarization mediated contractility in male and OVX rats might be the difference in the Ca^{2+} influx of VSMCs. It has been reported that in male SHRs, the Ca^{2+} entry in VSMCs is greater than in their female counterparts (Crews *et al.* 1999). OVX in female rats, however, raises the Ca^{2+} influx to the level of male SHRs (Crews *et al.* 1999). It has also been suggested that estradiol-17β directly blocks Ca^{2+} channels and in this way inhibits depolarization (Collins *et al.* 1993).

6.6. Endothelium-dependent relaxations

In *Study III*, neither male gender nor OVX modified the endothelium-dependent relaxations. It has been reported that both gender and OVX alter the function of the endothelium. The basal release of NO is greater from the aortic rings of female rabbits than from those of male rabbits (Hayashi *et al.* 1992). OVX, however, diminishes the basal release to levels seen in male rabbits (Hayashi *et al.* 1992). In SHRs, OVX lowers both basal and shear stress-induced NO production (Huang *et al.* 1998). In this study, normotensive rats were used, which can partly explain the normal endothelium function.

The five-week soy protein supplementation in *Study IV* and the two-week genistein and estradiol-17β treatments in *Study V*, had no effect on the endothelium-dependent relaxations in SHRs. However, other investigators have shown that an oral genistein supplementation of four to five weeks improves endothelium-dependent relaxations in OVX rats (Squadrito *et al.* 2000). Estradiol-17β has a similar effect. An ERT of twelve weeks enhances endothelium-dependent relaxations in SHRs (Williams *et al.* 1988). The improved endothelial function is associated with the maintainance of NO synthesis by estrogen in the arterioles of OVX hypertensive rats (Huang *et al.* 1997). In *Studies IV* and *V*, the rats were still young at the end of the experiment and the treatment times were obviously too short to be able to affect the function of the endothelium.

6.7. Blood pressure

The soy protein supplementation, rich in genistein and daidzein, attenuated the development of hypertension in SHRs compared to the casein supplementation. Whether a soy-based diet could decrease blood pressure when hypertension has already developed was long time unclear. Quite recently, it has been shown that soy protein contains peptides, which inhibit ACE and thereby reduce blood pressure in SHRs (Wu & Ding 2001).

The protein source and the total amount may be important in the regulation of blood pressure. Epidemiological studies suggest an inverse relationship between dietary protein intake and blood pressure, but published intervention trials do not support this hypothesis (for review, see Nurminen *et al.* 1998). In *Study IV* the total protein content of the soy protein used was 90% and the total protein content of casein was 86%, and the total protein contents of soy-based and casein-based diets were 18% and 17% respectively. This difference is too small to explain the lower blood pressure in SHRs on soy-based diet.

In monozygotic twins, a direct association of dietary protein intake and diastolic blood pressure has been described; the more protein-rich their diet, the greater the increase in their diastolic blood pressure (Havlik *et al.* 1990). In *Study IV*, only systolic blood pressure was measured. Therefore the relationship between protein supplementation and diastolic blood pressure remains to be clarified. Although dietary casein, an animal protein derived from milk, accelerated the development of hypertension in SHRs in *Study IV*, its effect is not always detrimental to experimental hypertension. A casein-rich diet has been found to attenuate the development of severe hypertension in stroke-prone SHRs (Ikeda *et al.* 1987).

The soy protein used in *Study IV*, contained genistein and daidzein. In *Study I* and *II* we showed that genistein and daidzein relaxed arterial smooth muscle endothelium-indepedently. Peripheral arterial resistance regulates blood pressure. It is possible that genistein and daidzein reduce peripheral resistance and thus attenuate the development of hypertension.

Two-week treatments with estradiol-17β, or low- or high-dose genistein treatments did not prevent the development of hypertension in OVX SHRs. However, in the normal SHRs, ERT of nine weeks (Hoeg *et al.* 1977) or seven months (Iams & Wexler 1979) lowers blood pressure. In OVX female SHRs, an ERT of seven months attenuates the development of hypertension (Iams & Wexler 1979). In the present study, the blood pressure was unaffected, probably because of the relatively short treatment time.

To sum up, a soy protein diet protects against the development of hypertension, whereas casein is not beneficial in this respect in SHRs. However, it is not yet clear which component of the soy is the most important in the protection against high blood pressure.

7. CONCLUSIONS

The aim of the present study was to investigate the effects of the plant-derived estrogens genistein and daidzein on arterial tone and on blood pressure, and to compare their effects to those of estradiol-17β. The major findings and conclusions are as follows:

1. Genistein, daidzein and estradiol-17β relaxed arteries. Estradiol-17β was the most potent relaxant, and daidzein was the weakest.
2. Although genistein and daidzein bind to estrogen receptors (ER) and activate them, their relaxing effect on arteries was independent of the ERs. The relaxations were also independent of gender and the endothelium. These relaxations were mediated, at least partly, via K_{Ca}-channels, and this mechanism resembled that of estradiol-17β.
3. Genistein, but not daidzein, reduced tyrosine phosphorylation in the arterial rings and in cultured vascular smooth muscle cells, suggesting that the slightly more potent relaxing effect of genistein than daidzein may due to tyrosine kinase inhibition.
4. Male gender and ovariectomy increased the contractility of the resistance artery, which may be one risk factor of cardiovascular diseases in males and postmenopausal females.
5. Soy protein, rich in genistein and daidzein, attenuated the development of hypertension in spontaneously hypertensive rats compared to a casein-based diet. This indicates that the replacement of milk protein by soy protein may have a beneficial influence on blood pressure.
6. The two-day low-dose genistein treatment reduced the contractility of the renal arteries and the tyrosine phosphorylation of the arterial smooth muscle. Both these effects disappeared after the two-week genistein treatment.

In conclusion, the plant-derived estrogens genistein and daidzein relax arterial smooth muscle in a similar way as estradiol-17β in rat *in vitro*. These relaxations are related to the activation of K_{Ca} channels, but are independent of endothelium, ERs and gender. The tyrosine kinase inbition by genistein also have a role in genistein-induced arterial relaxations. In addition, the soy protein has a beneficial effect on blood pressure. The present experimental findings agree with the epidemiological data, but controlled intervention studies are needed to verify these observations.

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